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STRUCTURE-ACTIVITY STUDIES OF PARATHYROID HORMONE AND COVALENT LABELLING OF A RENAL PARATHYROID HORMONE RECEPTOR

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Structure-Activity Studies of Parathyroid Hormone and Covalent Labelling of a Renal Parathyroid Hormone Receptor

A Thesis Submitted to Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

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Abstract

Section I: Four analogues of parathyroid hormone (PTH) containing D-amino acids were synthesized. Substitutions were made within the fully biologically active fragment of parathyroid hormone in the amino-terminal region, at position 2, and at the carboxyl-terminus, at position 34. The carboxl-terminal region contains structural determinants important to receptor binding. The amino-terminal region plays a critical role in the hormone-stimulated activation of adenylate cyclase **in vitro** and the expression of hormonal activity **in vivo**. Placement of a D-amino acid at the carboxyl-terminus yielded an analogue, [D-Tyr^{34}]bPTH-(1-34)-amide, 270% as active in the **in vitro** renal adenylate cyclase assay as unsubstituted bPTH-(1-34). In contrast, placement of a D-amino acid in the amino-terminal region, as in the analogues [D-Val^{2}, D-Tyr^{34}]bPTH-(1-34)-amide and [D-Val^{2}, D-Tyr^{34}]bPTH-(2-34)-amide, resulted in a nearly complete loss of biological activity. Deletion of a single residue at the amino-terminus, as in the analogue [D-Tyr^{34}]bPTH-(2-34)-amide, also caused near total loss of biopotency. These marked declines in biopotency occurred despite the presence of activity-enhancing modifications at the carboxyl-terminus of the latter three analogues. The most potent of the analogues, [D-Tyr^{34}]bPTH-(1-34)-amide, sustained an apparently spontaneous and complete loss of biopotency over a period of several weeks. Detailed studies of the mechanism of inactivation revealed an unusual lability of methionine residues to oxidation. Reduction under controlled conditions restored nearly completely both the methionine content and biological activity.

In order to avoid the oxidation-caused loss of biopotency three sulfur-free analogues of parathyroid hormone containing D-amino acids were synthe-

sized. Norleucine, a sulfur-free non-natural amino acid isosteric with methionine, was used. All three analogues were compared **in vitro** in a renal adenylate cyclase assay, a parathyroid hormone receptor binding assay, and **in vivo** in the chick hypercalcemia assay. The analogue [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide was found to be 440% as potent as unsubstituted bPTH-(1-34) making this the most potent analogue yet synthesized. The enhanced potency was largely attributable to increased affinity for the parathyroid hormone receptor. **In vivo**, however, this analogue was only 34% as potent as bPTH-(1-34). Cumulative evidence suggests that the 12-fold decline in the relative potency when the compound was assayed **in vivo** is due to the substitution of norleucine for methionine. The other analogues, [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide and [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide, were only weakly biologically active in the **in vitro** renal adenylate cyclase assay and receptor binding assay and **in vivo** indicating that substitution with D-amino acids at the amino-terminus of PTH results in a markedly diminished receptor affinity. The placement of a D-amino acid at the amino-terminus is more deleterious to biological activity than is the ommision of amino acids at positions 1 and 2. These results suggest useful directions for further structure-activity studies with parathyroid hormone.

Section II: Canine renal cortical membranes are presumed to contain a parathyroid hormone-specific receptor: PTH stimulates adenylate cyclase in these membranes and competition for binding sites by a series of hormone fragments and analogues has been demonstrated in a radioreceptor assay using these membranes. To identify a PTH receptor component in these membranes, the sulfur-free synthetic analogue of PTH, [Nle^{8}, Nle^{18}, Tyr^{34}]-

bPTH-(1-34)-amide, was radiolabelled with ^{125}I. Two photolabile aromatic azide compounds, 4-Fluoro-3-Nitrophenyl azide and N-Succinimidyl-6(4'-Azido-2'-Nitrophenyl Amino) Hexanoate, were separately conjugated through lysines to different preparations of ^{125}I-labelled analogue in the dark. Concurrently, a corresponding ^{127}I-labelled analogue was prepared, HPLC purified, and demonstrated to be fully biologically active in the **in vitro** renal adenylate cyclase assay. Both radiolabelled photolabile analogues were incubated in darkness with canine renal membranes for 45 minutes, in the presence or absence of competing hormone, and then exposed to light from a high-pressure mercury source to covalently link the analogues to membrane components. Membranes were then solubilized and fractionated on SDS-polyacrylamide gels. Radioautographs of the gels were made. In the absence of competing hormone, 4-6 membrane components were labelled by each of the photolabile analogues. However, only one band labelled specifically: in the presence of competing hormone, a membrane component failed to label. The position of this band (MW = 70,000) was identical regardless of which photolabile analogue was used. This same band labelled in the presence of inactive fragments of PTH, but not when competing PTH agonist or antagonist analogues were present. Additionally, a porcine renal cell line membrane known to have calcitonin-specific adenylate cyclase activity but no PTH-specific activity, was incubated and reacted with the photolabile compounds with and without competition. Almost no labelled bands were evident, there were no changes in the pattern with competition, and the radioautographs lacked a 70,000 MW band. These studies identify and will facilitate harvesting and further characterization of the PTH renal receptor or receptor-subunit.

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Acknowledgments

One of the pleasures (some would say the only one) of writing a thesis or any lengthy tome is being able to thank, in black and white, some of the people who helped so much. Dr. Joseph Majzoub I would like to thank for his patience in initiating me in the mysteries of gels and radioautographs and his unflagging interest in my progress. To Gary Shepard, whom I owe so much for massive amounts of help during times I needed five hands and only had three, I extend a heartfelt well done. Dr. John Potts, Jr., I want to thank for the ideas and dialogues we exchanged over these past three years but most of all I want to pay tribute to his assessment of character and abilities in matching up people, traits I've come to admire and appreciate. And finally to Dr. Michael Rosenblatt, who to me embodied all the above, patience, help, and judgement, I say thanks, especially for giving me the chance and opportunity that someone once gave him. I certainly hope my path crosses theirs again.

Introduction

Structure-activity relationships have been an important area of investigation in hormone research. Through modifications of the naturally occurring parent molecule, both enhanced-potency agonist and antagonist analogues have been prepared. Indirectly, information about the hormone receptor has been obtained in this manner. Such has been the case with parathyroid hormone (PTH). Figure 1 depicts the sequences of the three parathyroid hormones that have been studied in any detail: bovine, human and porcine. Historically, bovine parathyroid hormone (bPTH) occupies the most prominent position. First extracted in 1925 from bovine parathyroid glands, bPTH has always been the most available owing to its slaughterhouse source. In contrast, human parathyroid hormone (hPTH) was originally available primarily from adenoma tissue removed during surgery. The full structure of human parathyroid hormone was only elucidated in 1978 (20) while the bPTH sequence has been known since 1970 (4,33). As a consequence, almost all the structure-activity research has been on bPTH and continues to be. This thesis concerns itself with the preparation and evaluation of bPTH analogues in search of the super-potent analogue, definition of the molecular "elbow room" in the receptor, and ultimately the characterization of the receptor itself. The first two goals involved the preparation of a class of parathyroid hormone analogues incorporating D-amino acids. The last goal made use of a related but separate methodology to prepare a biologically active photolabile bPTH analogue which was used to tag and subsequently separate out the PTH membrane receptor. To adequately discuss these two developments, they will be treated separately in two complete sections.

This thesis summarizes my work over the past three years in the Endocrine Unit at Massachusetts General Hospital. I originally sent a letter to Dr. John Potts, Jr., head of the unit, inquiring into the possibility of working on a synthetic biochemical project for the summer between my first and second years at Yale. My letter was forwarded to Dr. Michael Rosenblatt, the man I've been associated with these three years. Dr. Rosenblatt, who had been working on analogues of parathyroid hormone, was thinking of the incorporation of D-amino acids in PTH at that time. With my undergraduate background in chemistry, the project was given to me. Out of this first summer's work, spanning three and a half months, and numerous discussions long after I left, two papers resulted: Analogues of Parathyroid Hormone Containing D-Amino Acids: Evaluation of Biological Activity and Stability, Coltrera, Rosenblatt, & Potts, Biochemistry (1980) 19, 4380-4385, and; Sulfur-free Parathyroid Hormone Analogues Containing D-Amino Acids: Biological Activity **In Vitro** and **In Vivo**, Rosenblatt, Coltrera, Shepard, Gray, Parsons, & Potts, submitted to Biochemistry 1/81. In addition, the work covered in the first paper was presented at the Endocrine Society meeting in 1979. All the proceedures outlined in Section I were taught to me by different people in the Endocrine Unit and performed by myself or with the help of others except for the amino acid analyses and the **in vivo** chick hypercalcemia assay which was done by Dr. John Parsons, England. The work on the sulfur-free analogues was completed, using my peptides, after I had returned to Yale for my second year.

The successful outcome of the original work encouraged me to tackle the more complex problem of finding and characterizing the renal receptor for parathyroid hormone. Done over the past six months, the second section deals with this work and includes the techniques and protocols developed by

me for the syntheses of photolabile PTH analogues and the labelling of the PTH receptor. Other techniques new to me were, once again, picked up from others in the Endocrine Unit and performed by myself or with help.

Aside from the successful outcome of my research projects, my association with the Endocrine Unit and Drs. Rosenblatt and Potts in particular has been a happy one. I've been exposed to many research techniques as well as an invaluable methodology for approaching problems in the medical field which I know will stand me in good stead in my future career.

SECTION I

Analogues of Parathyroid Hormone Containing D-Amino Acids

Part I:

Substitution of D-amino acids for their naturally occurring L-amino acid brethren has produced analogues of several peptide hormones and other biologically active peptides that are considerably more potent than their native counterparts (5,7,12,15,18,25,29,34,43,50,51,52,61,62). The enhanced bioactivity observed for these D-amino acid-containing analogues may be the result of one or more mechanisms. Analogous to a key in a lock, alterations of the hormonal conformation produced by the inclusion of D-amino acids may change the interaction between hormone and receptor (54,63), providing a better fit. On the other hand, D-amino acids may make the hormone analogue more resistant to enzymatic degradation thus prolonging its survival time and availability to the receptor (9,24,36,44). In addition, in the **in vivo** system where renal and other clearance times become a factor, a D-amino acid-containing analogue might have a different clearance time than the naturally occurring hormone with a subsequent effect on circulating hormone levels and availability.

For bovine parathyroid hormone (bPTH) (Figure 2), the amino acid sequence 1 to 34 (starting at the amino terminus) has been found to have the structure necessary for the full biological activity of the parent molecule, bPTH(1-84) (39,41,59). Further it has been shown that the minimum sequence needed for biological activity is 2 to 26 (58). In substituting 1 to 34 particular attention has been payed to the amino- and carboxyl-terminal regions. At both of these areas, marked alterations in bioactivity have occurred when these structures have been modified. In particular, certain modifications at positions 1 or 34 can produce up to a three-fold increase

in biological activity **in vitro** and **in vivo** (39,45). Of these, the most successful modification to date has been the substitution of tyrosine for the phenylalanine in position 34 plus the modification of the carboxyl-terminal to an amide ($CONH_2$). With these facts in mind, an examination of the effects on the biological activity by D-amino acid placement in either the amino- or carboxyl-terminal region of bPTH(1-34) was undertaken. Four D-amino acid containing hormone analogues were synthesized by the Merrifield solid-phase method (30,31,32): [D-Tyr^{34}]bPTH-(1-34)-amide, [D-Val^2, D-Tyr^{34}]bPTH-(1-34)-amide, [D-Tyr^{34}]bPTH-(2-34)-amide, and [D-Val^2, D-Tyr^{34}] bPTH-(2-34)-amide. A single synthesis was branched at the appropriate points to produce the four peptides thus minimizing variations between them. The biological activity of the analogues was evaluated in the **in vitro** rat renal cortical adenylate cyclase assay (21,26,27).

Materials and Methods

Synthesis and Purification. Four analogues of bovine parathyroid hormone, [D-Tyr^{34}]bPTH-(1-34)-amide, [D-Val^2, D-Tyr^{34}]bPTH-(1-34) amide, [D-Val^2, D-Tyr^{34}]bPTH-(2-34)-amide, and [D-Tyr^{34}]bPTH-(2-34)-amide were prepared by a modification (10,46,48) of the solid-phase method of Merrifield (30,31,32). The primary structure of the analogues is depicted in Figure 3. Synthesis was performed manually in a 50 ml plastic syringe fitted with a Luer-Lok drain and a porous polyethylene filter disc. A mechanical stirrer fitted with a Teflon-coated stirring rod was fixed above the reaction vessel. Benzhydrylamine resin (polystyrene-1% divinyl-benzene, Beckman) was used as the solid support and to effect the carboxy-

amide ($CONH_2$) carboxyl-terminal modification.

The **tert**-butoxycarbonyl (Boc) group was used to protect α-amino groups during coupling, except for arginine, which was protected by an amyloxycarbonyl group. Amino acid side-function protection was obtained as follows: (a) the serine hydroxyl group was protected as the **O**-benzyl ether; (b) the tyrosine hydroxyl group was protected as the **O**-2,6-dichlorobenzyl ether; (c) the carboxyl group of aspartic and glutamic acids was protected as the benzyl ester; (d) the histidine imidazole nitrogen and the arginine guanidine function were protected by the **p**-toluenesulfonyl group (histidine was stored as the dicyclohexylamine salt and desalted immediately before use); (e) the lysine ϵ-amino group was protected by a 2-chlorocarbobenzoxy group. L-Amino acids were obtained from Beckman Instruments and Peninsula Laboratories; D-amino acids were obtained from Bachem Chemicals and Peninsula Laboratories.

D-Tyrosine was coupled to the benzhydrylamine resin to begin the synthesis. Five grams of the copolymer resin (usable nitrogen: 0.51 meq/g) was washed (6x) with methylene chloride (CH_2Cl_2) (distilled over potassium carbonate) then treated with 30% (v/v) trifluoroacetic acid (TFA) (Pierce) in methylene chloride (2x 2 min then 30 min). The resin was then neutralized with 10% (v/v) triethylamine (Pierce) in methylene chloride (2x 2 min then 10 min) followed by washing (4x) with methylene chloride. Coupling of the amino acid was done by adding a 2.5 molar excess of BOC-**O**-2,6-dichloro-benzyl-D-Tyrosine and a 2.5 molar excess of dicyclohexylcarbodiimide in methylene chloride and stirring for 3 h. The amino acid-copolymer resin complex was then washed (6x) with methylene chloride. The unreacted co-polymer benzhydrylamine groups were then acetylated irreversibly by adding 2.5 ml acetic anhydride and 0.75 ml triethylamine in 25 ml DMF (20 min).

Subsequent amino acids were added in a like manner with the exception of arginine and glutamine which were coupled by the "active ester" method. For these two amino acids, a 5 molar excess of their respective **p**-nitrophenyl "active ester" in dimethylformamide was added, stirred 2 h and left to react overnight. Figure 4 schematically outlines these steps. After incorporation of tryptophan at position 23, 1% mercaptoethanol was added to the trifluoroacetic acid reagent and to the TFA-associated methylene chloride washes in all subsequent steps in order to minimize oxidation (28).

Couplings were monitored qualitatively for completeness by the fluorescamine test (11). Double couplings were needed to obtain a negative fluorescamine test after the addition of glutamine at 29, leucine at 28, lysine at 27, tryptophan at 23, glutamic acid at 22, arginine at 20, and methionine at 8. Triple couplings were needed for leucine at 24 and arginine at 25.

Prior to cleavage, the peptide-resin complex was washed with 30% TFA w/1% mercaptoethanol, 1% mercaptoethanol/CH_2Cl_2, CH_2Cl_2 (5x), and then shrunk with ethanol and lyophilized. Anhydrous hydrogen flouride (HF) was used to simultaneously cleave the peptide from the resin and remove the side-chain protecting groups. An excess of doubly-distilled hydrogen fluoride was added to the peptide-resin complex along with 7 ml distilled anisole (3.5 ml/2.5 g resin) and the reaction was run at 0°C for 1 h. Hydrogen fluoride was then removed by reduced pressure distillation. The peptide was then extracted by alternating glacial acetic acid and water washes (4x each). The washes were combined and lyophilized.

The crude peptide was taken up in 1 M acetic acid and mounted first on a Bio-Gel P-6 column (2.0 x 100 cm) (Bio-Rad Labs). Eluted with 1 M acetic acid, the major peak was collected, lyophilized and applied to a carboxy-

methylcellulose ion exchange column (CM-52, 1.2 x 15 cm) (Whatman) in the presence of 8 M urea. An **LKB Ultragrad** apparatus was used to create a shallow sloped conductance gradient from two ammonium acetate buffers: 1.5 mmho (pH 5.1) and 20.0 mmho (pH 6.2). The top of the largest peak was placed on a Bio-Gel P-2 column (Bio-Rad Labs) for separation of the peptide from the urea and ammonium acetate.

Analytic Methods. Amino acid analyses were conducted with a Beckman Model 121 M-B automated analyzer. Acid hydrolysis was performed in 5.7 N HCl at 110°C in an evacuated dessicator for 24 h in the presence of 1:2000 (v/v) mercaptoethanol to protect the methionine residues (19). Total enzymic digestions were performed by using papain (enzyme/substrate ratio 1:50, pH 5.4, 2 h, 37°C), followed by aminopeptidase M (Henley and Co.) (enzyme/substrate ratio 1.5:1.0, pH 8.2, 3 h, 37°C) in order to correctly analyze the labile residues of asparagine, glutamine and tryptophan (41).

The peptides were analyzed by thin-layer chromatography and thin-layer electrophoresis. Two thin-layer chromatography systems using precoated cellulose plates (100um, Brinkmann) and ninhydrin staining were employed: (A) butanol/pyridine/acetic acid/water (15:10:3:12); (B) pyridine/acetic acid/water (30:1:270). Two thin-layer electrophoretic systems using cellulose-coated plates (100um) and ninhydrin staining were employed: (A) pyridine/acetic acid/water (30:1:270), pH 6.5 (600 V, 11mA, 1.5 h); (B) 2% formic acid and 8% acetic acid, pH 2.0 (600 V, 5-8 mA, 45 min). Sequence analyses were performed to quantitate contamination by deletion-containing error peptides as well as to confirm the correct amino acid sequence (56,60). Reverse-phase high-pressure liquid chromatography was performed with a C^{18} uBondapak column (Waters Associates), two buffers (buffer 1, 20% aceto-

nitrile and 80% water with 0.1% trifluoroacetic acid throughout; buffer 2, 50% acetonitrile and 50% water with 0.1% trifluoroacetic acid throughout), a flow rate of 1.5 ml/min, and a linear gradient of 0-100% buffer 2 over 20 min (2).

Bioassay. Assessment of biological activity **in vitro** was performed using a modification of the rat renal cortical adenylate cyclase assay (21, 26,27). The incubation mixture consisted of the following: 0.05 M Tris-HCl 5.4 mM theophylline, 5.4 mM $MgCl_2$, 7.8 mM KCl, 0.011% (w/v) bovine serum albumin, 0.75 mM ATP, 2 x 10^6 cpm of [α-^{32}P]ATP, 4.5 mM creatine phosphate, 0.075 mg/ml creatine phosphokinase, and 50 to 100 ug of membrane protein. The peptides were added in 5 ul and the total volume was brought to 100 ul. Incubations were started by the addition of the membrane suspension and run for 10 min at 37°C. Termination was accomplished by the addition of 100 ul of a solution consisting of 0.12 M unlabeled cyclic AMP (cAMP), 0.05 M ATP and approximately 20,000 cpm of [^{3}H]cAMP in 0.05 mM Tris-HCl. [α-^{32}P]ATP and [^{3}H]cAMP were obtained from New England Nuclear. The bPTH standard used in the assays was Medical Research Council Standard, lot no. MRC 72/286. Each preparation, except [D-Tyr^{34}]bPTH-(1-34)-amide, was assayed at least 3 times at multiple concentrations within 4 weeks of completion of purification. Preparations were not treated with reducing agents prior to assay, except as described below for a single preparation of [D-Tyr^{34}]bPTH-(1-34)-amide. The individual potency estimates were combined to yield the mean potency of each analogue. For [D-Tyr^{34}]bPTH-(1-34)-amide, a combined potency determination was not possible because of the observed instability of biological activity.

Reduction of Oxidized [D-Tyr^{34}]bPTH-(1-34)-amide. [D-Tyr^{34}]bPTH-

(1-34)-amide (400ug), which had declined to an undetectable level of bioactivity (presumably due to oxidation), was reduced by treatment with 1.0 ml of 0.1 M ammonium acetate (pH 7.4) that was 2 M in mercaptoethanol for 20 h at 37°C (65). After treatment, the solution was diluted with 2.0 ml of water, placed on a Bio-Gel P-2 column (Bio-Rad) for desalting, then lyophilized and immediately assayed.

Results

Analytical Data. Amino acid analysis of each of the four peptides is presented in Table I. No heterogeneity of the purified peptides was detected in the thin-layer chromatographic or electrophoretic systems used. Analytical data for one of the four peptides obtained from the single synthesis, [D-Val^2, D-Tyr^{34}]bPTH-(1-34)-amide, are provided. The peptide had an R_f = 0.62 in TLC system A and an R_f = 0.81 in system B. Electrophoretic mobility relative to leucine was 0.8 in thin-layer electrophoretic system A and 1.43 in system B. Accumulated preview† was determined through 30 cycles of automated Edman sequence analysis: the purified peptide contained no more than 4% contamination by deletion-containing error peptides (56,60). A high-pressure liquid chromatographic profile of 20 ug of [D-Val^2, D-Tyr^{34}]bPTH-(1-34)-amide is shown in Figure 6. Only a small amount of heterogeneity was revealed; contaminants were estimated to be <5%.

Bioactivity. Figure 7 depicts stimulation of rat renal cortical membranes in the adenylate cyclase assay by native bPTH-(1-84) (used as the assay standard) and the four D-amino acid-containing analogues. [D-Tyr^{34}]-bPTH-(1-34)-amide is more potent than native bPTH-(1-84). The other analogues

† For an explanation of accumulated preview see Figure 5.

are only weakly biologically active, and their dose-response curves are non-parallel to the curve generated by the native hormone standard, indicating a qualitative difference in the nature of the observed adenylate cyclase stimulation. Mean potencies for each of the analogues are listed and compared with the potency of unsubstituted bPTH-(1-34) and a previously synthesized (45) highly active PTH analogue, [Tyr^{34}]bPTH-(1-34)-amide, in Table II. Due to the instability of the analogue [D-Tyr^{34}]bPTH-(1-34)-amide, the potency of 14,500 MRC units/mg obtained in the first assay of this analogue, 10 days after completion of purification, may actually be an underestimate of its true potency. An estimate of its initial potency may be derived from an extrapolation of the decreasing biopotencies back to the time of synthesis completion. The potency at that time might have been 18,000 MRC units/mg or greater.

Instability of [D-Tyr^{34}]bPTH-(1-34)-amide. A progressive decline in biological activity was observed for the analogue [D-Tyr^{34}]bPTH-(1-34)-amide. The peptide had been stored as a lyophilized powder in a closed vial in the dark at room temperature. Within 18 weeks of completing the synthesis the biopotency had fallen from 14,500 MRC units/mg to an undetectable level. Intermediate potencies are plotted as a function of time from purification completion in Figure 8.

Accompanying the loss of biological activity was a complete loss of methionine content as determined by amino acid analysis after enzymatic hydrolysis. No methionine sulfone was detected at 18 weeks, indicating probable spontaneous oxidation of the methionines to the intermediate oxidation state of methionine sulfoxide. Reduction of the inactive material led to a restoration of 90% of the methionine content and a

concomitant restoration of most of the bioactivity. The potency of the reduced material was 11,100 MRC units/mg.

Part II: Sulfur-Free Analogues

The instability of [D-Tyr^{34}]bPTH-(1-34)-amide and its consequent rapid decline in biopotency precluded an accurate **in vivo** assay of the compound. Since the mechanism of inactivation was found to be the unusual susceptibility of the two methionine residues (at positions 8 and 18) to oxidation, a stable analogue using a substitute for methionine was needed. Norleucine, a non-natural, sulfur-free amino acid has been substituted for methionine previously (46).

CH_2-S-CH_2-CH_2-CH(NH_2)COOH
Methionine

CH_2-CH_2-CH_2-CH_2-CH(NH_2)COOH
Norleucine

Nearly isosteric with methionine, norleucine was found to be well-tolerated in terms of biopotency **in vitro**. The analogue [Nle^8, Nle^{18}, Tyr]bPTH-(1-34)-amide had an **in vitro** biopotency 76% that of unmodified bPTH-(1-34). The analogues were stable in long-term storage at room temperature.

Sulfur-free versions of three of the analogues containing D-amino acids were synthesized by the Merrifield solid-phase method (30,31,32): [Nle^8, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, [D-Val^2, Nle^8, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, [D-Val^2, Nle^8, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide. As before, a single synthesis was branched to create the three peptides to permit the most valid comparision of biological activity. Biological properties were evaluated in the **in vitro** renal adenylate cyclase assay (21,26,27), the PTH-specific renal radioreceptor assay (53), and the **in vivo** chick hypercalcemia assay (38).

Synthesis and Purification. The three sulfur-free analogues of bPTH, [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide were prepared manually as previously described (see page 2). The primary structure of the analogues is depicted in Figure 9. Couplings were monitored qualitatively for completeness by the fluorescamine test (11). Double couplings were required to obtain a negative fluorescamine test after addition of lysine at 26, leucine at 24, and isoleucine at 5. Triple couplings were required for glutamine at 29 and asparagine at 10. After double couplings of asparagine at 33, a positive fluorescamine test was obtained. At this point, the peptide resin was acetylated to terminate any unreacted peptide chains. Purification of the peptides was performed by gel-filtration followed by ion-exchange chromatography, as previously described (see page 4).

Analytic Methods. Amino acid analyses as well as thin-layer chromatography (TLC) and thin-layer electrophoresis (TLE) were performed as described in Part I (see page 5) with the exception of the TLE systems: (A) pyridine:acetic acid:water (30:1:270), pH 6.5; (B) pyridine:acetic acid water (1:10:289), pH 3.5. Sequence analyses were again done to quantitate contamination by deletion-containing error peptides as well as confirm the correct amino acid sequence (56,60). Reverse-phase high-pressure liquid chromatography was performed using a uBondapak column (Waters Associates), two buffers (Buffer 1: 20% acetonitrile and 80% water-0.1% trifluoroacetic acid throughout; Buffer 2: 90% acetonitrile and 10% water-0.1% trifluoroacetic acid throughout), flow rate 1.5 ml/min and a linear gradient of

0-100% Buffer 2 over 20 minutes (2).

Bioassays

Rat Renal Adenylate Cyclase Assay. Assessment of biological activity **in vitro** was performed using a modification of the rat renal cortical adenylate cyclase assay as in Part I (see page 6). The bPTH standard used in the assays was Medical Research Council Standard, Lot #MRC 72/286. Each preparation was assayed at least three times at multiple concentrations and the separate potency estimates were combined to yield the mean potency of each analogue.

Radioreceptor Binding Assay. The parathyroid hormone renal receptor binding assay is based on canine renal cortical membranes and a sulfur-free radioiodinated PTH analogue ligand ^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide (53). Protocols for the preparation of the dog renal cortical membranes and the ^{125}I-bPTH analogue may be found on pages 25 and 23 respectively. 100 to 175 ug of membrane protein and 20,000 to 30,000 cpm of ^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide were incubated in a reaction media consisting of 0.05 M Tris-HCl, 9 mM theophylline, 4.2 mM $MgCl_2$, 26 mM KCl, 0.118% (w/v) bovine serum albumin, and 5% (v/v) parathyroidectomized dog serum for a total volume of 250 ul. The incubations were run at 15°C for 60 min and the test tubes were gently vortexed for a few seconds every 15 min. 200 ul was taken from each test tube and placed in 400 ul plastic microfuge tubes (Beckman Instruments, Inc., Scientific Instruments Division) which were then spun down at 8000 x g in a Beckman microcentrifuge Model 152 (Beckman Instruments, Inc.) for 3 min. The supernatant, containing the unbound radioactive ligand, was drawn off by a 22 guage lumbar-puncture needle. The tips of the microcentrifuge

tubes, containing the spun-down membrane and its bound component of radioactive ligand, were cut off and counted for ^{125}I in a Packard 8-well spectrophotometer (Packard Instrument Co.). In order to determine the total radioactivity added to each incubation tube, controls were run without membranes and, after incubation, a 200 ul aliquot was transferred from control to microfuge tube and counted. The 200 ul control aliquots were then aspirated and the microfuge tubes were counted for adherence of the radioligand to the plastic tube. Losses during incubation were assessed as 50 ul and this amount was also counted. The total losses were found to be constant at 10 ± 2% (S.E.M.).

Each analogue was tested over a concentration range of 1×10^{-9} M to 1×10^{-4} M and was assayed at least three times.

In Vivo Chick Hypercalcemia Assay. The intravenous chick hypercalcemia assay (38) was used to assess **in vivo** biological properties.

Results

Analytical Data. Amino acid analysis of each of the three peptides is presented in Table III. No heterogeneity of the purified peptides was detected in the thin-layer chromatographic or electrophoretic systems employed. Analytical data for one of the three peptides obtained from the single synthesis, [D-Val^2, Nle^8, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, is provided. The peptide had an R_f = 0.69 in TLC system A and an R_f = 0.8 in system B. Electrophoretic mobility relative to lysine was 0.71 in TLE system A and 0.89 in system B. Accumulated preview† was determined through 30 cycles of automated Edman sequence analysis: the purified peptide contained less than 4% contamination by deletion-containing error peptides. A

† For an explanation of accumulated preview see Figure 5.

high-pressure liquid chromatographic profile of 20 ug of [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide is shown in Figure 10. Heterogeneity is estimated to be <2%

Bioactivity. Figure 11 depicts stimulation of rat renal cortical membranes in the adenylate cyclase assay by native bPTH-(1-84) (used as the assay standard) and the three analogues. [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide is more than four times as potent as native bPTH-(1-84). The weighted mean potency of 24,000 MRC units/mg makes this peptide the most potent PTH analogue yet synthesized. The other analogues were only weakly biologically active and the dose response curves obtained for each of these analogues are non-parallel to the curve generated by the native hormone standard, indicating a qualitative difference in the nature of the observed adenylate cyclase stimulation. Mean potencies for each of the three analogues are listed and compared with the potency of unsubstituted bPTH-(1-34) in Table IV.

In vivo biopotencies determined in the chick hypercalcemia assay are also listed in Table IV. For the analogue [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, there is a marked disparity between **in vitro** and **in vivo** biopotency relative to the unsubstituted bPTH-(1-34). Although this analogue has enhanced potency **in vitro**, approximately 440% as active as bPTH-(1-34), the compound is only 34% as active as bPTH-(1-34) **in vivo**. This represents an approximately 12-fold decline in biopotency in the transition from **in vitro** to **in vivo** bioassay systems.

The remaining two analogues, [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide and [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide, are comparably weak in bioactivity **in vitro** and **in vivo**. The weak potency

of these compounds can be directly attributed to the decreased affinity for the PTH receptor, as demonstrated in the renal receptor binding assay, Figure 12. In contrast, [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide had an increased affinity for the receptor.

Discussion

Up until now, structure-activity studies of parathyroid hormone have followed two general directions. The first has been the identification of the functional segments of bPTH and the second has been the substitution of naturally occurring amino acids at different positions in the native sequence.

Previous studies have ascertained that the minimum sequence needed for full biological activity **in vivo** and **in vitro** is 1 to 34 (39,41,59). This minimum sequence was found to be further resolvable. The region comprising positions 1 and 2 at the amino-terminus is necessary for the activation of adenylate cyclase once receptor binding had occurred. Distinct from the activation region is the sequence 3 to 34 which is largely responsible for the binding to the presumed parathyroid receptors (14,47,49). The sequence 3 to 34 forms the basis for the present inhibitor designs. Figure 13 illustrates these regions in bPTH. The terminal sequence 25 to 34 has been found to contain the structural features most important for the receptor binding(35).

Substitution of naturally occurring amino acids at position 1 in the 1 to 34 sequence by Tregear & Potts (57) has shown that even subtle alterations in the structure there led to large variations in the biopotency of bPTH. Generally, position 1 alterations represent decreases in bioactivity. Certain modifications at the carboxyl-terminus, the phenylalanine at posi-

tion 34, yielded analogues of enhanced activity. Substituting tyrosine for phenylalanine produced an analogue 140% as active as unsubstituted bPTH-(1-34) (46). The modification represents the addition of one hydroxyl group on the phenyl ring. Converting the carboxyl-terminal carboxylic acid function [-COOH] to a carboxyamide [$-CONH_2$] produced an analogue which was 270% as potent as bPTH-(1-34) (39). The analogue [Tyr^{34}]bPTH-(1-34)-amide, which combines both of these modifications, was found to be almost 300% as active as bPTH-(1-34) (45).

The approach chosen here was to incorporate, for the first time, non-natural amino acids to pursue another possible design form for analogues. While the carboxyl-terminal binding region had tolerated modifications well in the past, modifications at the amino-terminus (the activation region) had shown the position 1 alanine to be a highly critical feature. Therefore position 2, valine, was chosen as the best sight for D-amino acid substitution in the amino-terminal region.

The rat renal adenylate cyclase assay was chosen for biological evaluation of all the analogues **in vitro**. The dog renal assay was also available for the evaluation of the sulfur-free analogues but there were distinct advantages to using the rat renal assay again. First, this choice allowed direct comparison with the original sulfur-containing D-amino acid analogues. Secondly, the rat renal membrane preparation is relatively rich in enzymes that degrade PTH whereas the dog renal membrane preparation is cleaner and therefore more sensitive and thus is now the preferred assay. But part of the argument for trying D-amino acid incorporation was the possible resistance to enzymatic degradation this might confer on the molecule. The rat renal assay could be useful as a screening assay for such attributes. The highly purified dog renal membranes were used in the

renal radioreceptor assay. This assay provides a measure of the interaction of analogues with the PTH-specific renal receptors. To complete the biological evaluation with an **in vivo** study, the chick hypercalcemia assay was used. The hypercalcemia caused by intravenous PTH-like agonists is felt to be a reflection in large part of the biological action of PTH on the bone. In the past there has been a very close correlation between an analogue's **in vitro** and **in vivo** biological properties (39,58).

Studies reported here confirmed that modifications at the amino-terminal activation region were poorly tolerated in terms of biopotency. With D-Val in place of L-Val in position 2, there was a nearly complete loss of bioactivity in the **in vitro** adenylate cyclase system for both [D-Val^2, D-Tyr^{34}]bPTH-(1-34)-amide and [D-Val^2, D-Tyr^{34}]bPTH-(2-34)-amide. This loss of potency occurs in the face of the activity-enhancing D-Tyr^{34}-amide modification at the carboxyl-terminus. Simple deletion of position 1 in [D-Tyr^{34}]bPTH-(2-34)-amide resulted in a similar near total loss of biological activity (Table II).

Conversely, substitution of D-Tyr at the carboxyl-terminus of 1 to 34, is well tolerated. The analogue produced, [D-Tyr^{34}]bPTH-(1-34)-amide, is nearly equal in biopotency to the most active analogue produced before, [Tyr^{34}]bPTH-(1-34)-amide (Table II). Over a period of 18 weeks, however, the activity **in vitro** was completely lost corresponding to a progressive oxidation of both methionine residues (positions 8 and 18) in the molecule. Evaluation of the **in vivo** biological activity was not possible because of the instability of the molecule. Oxidation of methionine has been previously found to result in complete loss of PTH activity (37,40,42,55). Spontaneous decline in the biopotency of the larger native molecule bPTH-(1-84) has been seen to occur over a period of months to years (observed

in the Endocrine Unit, MGH). Similarly, other enhanced-potency analogues have lost differing amounts of bioactivity when stored in light protected containers, but the loss in [D-Tyr^{34}]bPTH-(1-34)-amide was much faster and more complete than any other. Back reduction of the methionines resulted in a regaining of 90% of the biological activity previously demonstrated. But it was obvious that, lacking a storage method utilizing reducing atmospheres or agents, the full biopotency of [D-Tyr^{34}]bPTH-(1-34)-amide had not been measured, the first assay being performed 10 days after purification.

For this reason sulfur-free, oxidatively-stable analogues containing D-amino acids were made. Once again, substitution of D-Val for L-Val in position 2 was poorly tolerated with an in vitro biopotency of <2% of bPTH-(1-34) for both [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide and [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide. **In vivo** assay of the two peptides also showed activity <2% that of bPTH-(1-34). These results could also be correlated with the observed decreased binding to the receptor (Figure 12).

In contrast, the analogue [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide was found to be more than fourfold as active as unsubstituted bPTH-(1-34) **in vitro** making it the most potent PTH analogue yet synthesized. This increased bioactivity **in vitro** correlated with an increased affinity for PTH-specific binding sites. The previous observation that [Nle^{8}, Nle^{18}]bPTH-(1-34) is only 45% as potent as bPTH-(1-34) **in vitro** (46) suggests the obvious generalization that the substitution of norleucine for methionine, while bestowing oxidative resistance on an analogue will also generally decrease their biopotency. If this is so, then [D-Tyr^{34}]bPTH-(1-34)-amide would be expected to have a biopotency even greater than [Nle^{8},

Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide and very likely significantly higher than originally estimated.

In vivo, norleucine substitution is more poorly tolerated. [Nle^{8}, Nle^{18}]bPTH-(1-34) has an activity **in vivo** of 1800 MRC units/mg† compared with a bPTH-(1-34) standard of 7700 MRC units/mg. [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide with its activity-enhancing carboxyl-terminus modifications has an **in vivo** activity of 2500 MRC units/mg†. [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide follows a similar pattern. **In vitro** it is more than 400% as potent as bPTH-(1-34) but **in vivo** it is only one-third as active as bPTH-(1-34). With an **in vivo** potency of 2600 MRC units/mg it still stands as the most potent of the norleucine containing analogues.

In comparison to [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, the two D-Val norleucine-containing peptides resulted in a decline in bioactivity. The two analogues, [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide and [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide, were <2% as active as bPTH-(1-34) **in vivo**. Apparently the altered conformation created by the D-amino acid placement in the amino-terminus is responsible for this decline. Furthermore, whereas the **in vitro** radioreceptor binding assay showed that placement of the D-amino acid at the carboxyl-terminus is actually affinity enhancing, placement of a D-amino acid in the amino-terminal region causes a decrease in receptor affinity of approximately 30-fold. In point of fact, placement of a D-amino acid in the amino-terminal region seems worse for bioactivity than the complete omission of the terminus (positions 1 and 2): the compound [Nle^{8}, Nle^{18},Tyr^{34}]bPTH-(3-34)-amide which is missing positions 1 and 2 is equal in binding affinity to [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide (35,53) and **in vivo** bPTH(2-34) is

†Parsons, J.A., Tregear, G.W., & Rosenblatt, M. unpublished results.

approximately 50% as potent as bPTH-(1-34) (39,58).

In summary, both **in vitro** and **in vivo** studies show that D-amino acid substitutions critically affect biological properties. Whether the effect is positive or negative depends on their position. The amino-terminus is virtually intolerant of such substitutions while the carboxyl-terminus tolerates them well. [Nle^{8}, Nle^{18}, $D\text{-}Tyr^{34}$]bPTH-(1-34)-amide with a biopotency of 24,000 MRC units/mg **in vitro** is the most potent PTH analogue yet made. But what may be an **in vivo** intolerance to norleucine has precluded an accurate **in vivo** appraisal of the hoped for benefits of D-amino acids. So, while placement of D-amino acids in the carboxyl-terminal region of the 1 to 34 fragment appears very promising, their **in vivo** evaluation must await either development of storage conditions to preserve the biological activity and methionine content or the synthesis of sulfur-free oxidatively-stable PTH analogues with higher **in vivo** bioactivities than the norleucine series.

SECTION II

Photoaffinity Labelling of the Parathyroid Hormone Receptor

As previously noted, PTH analogues can supply information about the membrane receptor in addition to their relative merits as synthetic replacements for native PTH-(1-84). This approach is an indirect one in that it gathers information without actually finding the receptor. However, the advantages to finding the receptor itself are obvious. In a highly purified form its biochemical structure as well as its physiologic properties would be easier to elucidate. Immunologic studies would be facilitated, especially the development of monoclonal antibodies, a current research problem. Purified receptor could also form the basis of a very sensitive radioreceptor assay. This section deals with the development of biologically active bPTH analogues used to tag the parathyroid hormone receptor in renal cortical membranes and the subsequent separation and identification of the receptor/receptor subunit from the membrane.

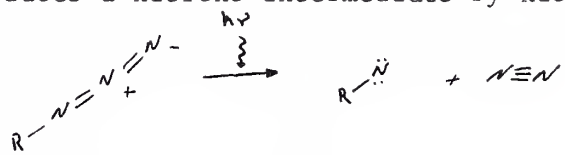
The Rationale

In approaching the problem of finding any receptor, hormonal or otherwise, the receptor must be tagged in an identifiable manner and this tag retained during the purification steps. Various approaches have been used, including radioactively labelled antibodies to a receptor, and gel columns impregnated with substances that have differing affinities for the receptor and other proteins. The approach that has been among the most versatile, and in the long run will probably prove to be the most successful, is photoaffinity labelling.

Basically, the problem of receptor labelling can be stated this way: the label must be highly specific for the receptor or the result would be useless. Few things have a greater specificity for the receptor than the

hormone itself, making PTH the ideal vehicle for tagging the receptor. With the advent of a radiolabelled, biologically active bPTH analogue, ^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide (46,53), a way to tag and follow the PTH receptor was available. If a covalent linkage could be formed between PTH and its receptor, then the hormone-receptor complex could be separated from the membrane and characterized by following the radioactive label. To achieve the linkage between hormone and receptor, a group capable of activation and reaction while the hormone is in the receptor must be attached to the hormone while retaining the hormone's bioactivity and specificity. Photoactivated groups have a distinct advantage in ease of activation while maintaining control over time and place. A number of very reactive groups are available and have been used to label several different receptor systems, most notably insulin, glucagon and acetylcholine (3,6,16,64).

The photoactive ligand must incorporate both the photolabile group used to effect the linkage between hormone and receptor, and a more conventional reactive group for attachment to the hormone. The most common hormonal attachment site has been a primary amine group. Attributes needed for the photolabile group itself include chemical stability before activation, activation energy absorptions at long enough wavelengths or short enough periods of time to preclude photochemical damage (i.e. oxidation) to the rest of the system, extremely short half-life, high reactivity, and resistance to intramolecular rearrangements which could result in less active forms (1). Among the choices for photolabile groups, aryl azides have proved to possess a good compromise of qualities. The azide, (N^3), upon activation produces a nitrene intermediate by kicking off nitrogen:



The nitrene, though less reactive than its carbon equivilent, the carbene, is capable of reacting with primary, secondary, and tertiary carbon-hydrogen bonds (C-H) as well as hydroxyls (O-H). Once activated, it will react with whatever is in its immediate proximity. Owing to the specificity of PTH, there is a high probability that the nitrene's neighboring proteins will include the receptor. Non-specific binding could be differentiated from specific binding through the use of cold hormone as competition with the photolabile analogue. Only the PTH-specific binding, i.e. the receptor, would be affected.

Two photolabile aryl azide-containing compounds were chosen for attachment to the ^{125}I labelled PTH analogue.

(1) 4-Fluoro-3-Nitrophenyl Azide (FNPA)
(2) N-Succinimidyl-6(4'-Azido-2'-Nitrophenyl Amino) Hexanoate (SMHA)

Both groups will react with primary amines under relatively mild conditions. The only available primary amine groups in 1 to 34 are the lysine ϵ-amino groups at positions 13, 26, and 27, and the position 1 alanine's α-amino group. Any addition to the position 1 alanine would probably result in an inactive analogue (see page 14) (57). But the ϵ-amino groups on lysine are normally much more reactive than the valine α-amino group and as long as the peptide's conformation does not totally block these groups the chances are good that this attachment will be the favored one.

The analogues, FNPA-^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide and SMHA-^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide, were evaluated separately with dog renal membrane preparations. High-pressure liquid chromatography purified (HPLC) FNPA-^{127}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide was assayed for biological activity in the **in vitro** dog renal adenylate cyclase assay.

Synthesis and Purification of [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide. The basic peptide was synthesized by the Merrifield solid-phase method and purified as previously outlined (see page 2).

Iodination. [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide was iodinated by a modified Hunter and Greenwood protocol (17). The reaction was run at room temperature in a 0.2 M sodium phosphate buffer (pH 7.5) using a molar ratio of hormone/I^{-}/Chloramine-T (Eastman Chemical) of 1:1.5:200. After 30 sec the reaction was stopped by the addition of sodium metabisulfite in a 2-fold molar excess to Cloramine-T.

For the radiolabelling of the peptide, $Na^{125}I$ (New England Nuclear) was used in a protocol calling for 1500 uCi per 2 ug of bPTH-(1-34) (46). 8 ug of [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide (1.9 nmol) was dissolved in 40 ul of 0.1 N HOAc and added to 200 ul of 0.2 N sodium phosphate buffer along with 6 mCi of $Na^{125}I$. Chloramine-T (380 nmol) in 40 ul buffer was added and allowed to react for 30 sec. The reaction was stopped with sodium metabisulfite (760 nmol) and a small amount was drawn off to check for ^{125}I incorporation. 1 ml of H_2O was added along with approximately 1 cc of QUSO-32 (Philadelphia Quartz Company) to adsorb the peptide. The sample was centrifuged and the supernatant was discarded. Excess ^{125}I was removed by the addition of 1 ml H_2O and I-resin, centrifuging and discarding the supernatant. 1 ml of 20%:1% acetone/glacial acetic acid was then added, the pellet resuspended, spun down and the supernatant drawn off and lyophilized.

A non-radioactive ^{127}I labelling of 2 mg [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide was done for use in the **in vitro** bioassay of the photo-

affinity compound. KI was used and trace amounts of ^{125}I were added to check for incorporation. After neutralization with sodium metabisulfite the reaction was lyophilized. The lyophilized product was then dissolved in <1 ml of 0.1 M HOAc in 8 M urea and placed on a Bio-Gel P-2 column (41 x 1.2 cm) previously equilibrated with 0.1 M HOAc. The peptide eluted in the void volume followed by Chloramine-T, metabisulfite, and iodine (Figure 14). The peptide was lyophilized and stored for further use.

Attachment of the Photolabile Groups. (1) 4-Fluoro-3-Nitrophenyl Azide (FNPA): 2 ug ^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide (478pmol) was dissolved in 100 ul DMSO. A 20-fold excess of FNPA (9.56 nmol) (Pierce) in 25 ul DMSO was added along with 1 x 10^{-9} mol triethylamine. The reaction was protected from light and agitated continuously at room temperature for 5 h. A 400-fold excess of glycine (191 nmol) was added to terminate the reaction.

(2) N-Succinimidyl-6(4'-Azido-2'Nitrophenyl Amino) Hexanoate (SMHA): 2 ug ^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide was dissolved in 100 ul 0.2 M sodium phosphate buffer, pH 7.5. A 20-fold excess of SMHA (9.56 nmol) (Pierce) in 20 ul buffer was added. The reaction was protected from light and agitated continuously for 1 h at room temperature. A 400-fold excess of glycine (191 nmol) was added to terminate the reaction.

The reaction products in both cases were stored at 4°C in the dark and used without further purification. The primary structure of the analogues is depicted in Figure 15 along with the photolabile groups.

FNPA-^{127}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide was also prepared in a scaled up FNPA synthesis for 2 mg of peptide for use in a representive

bioassay and analyses.

Analytic Methods. Reverse-phase high-pressure liquid chromatography was performed with a uBondapak column (Water Associates), two buffers (buffer 1, 5% acetonitrile and 95% water with 0.1% trifluoroacetic acid throughout; buffer 2, 90% acetonitrile and 10% water with 0.1% trifluoroacetic acid throughout), a flow rate of 2.5 ml/min, and a linear gradient of 0-100% buffer 2 over 30 min (2). Spectroscopic analysis was done with a Beckman Model 25 Spectrophotometer on material from the preparative HPLC.

Renal Cortical Membrane Preparation. Kidneys were obtained from healthy mongrel dogs weighing between 20 and 27 kg. The membranes were prepared following a modified method (53). The cortex was bluntly dissected from the medulla and homogenized at 4°C in a 30% buffer solution containing 0.25 M sucrose, 0.01 M Tris, and 0.001 M Na_2EDTA, pH 7.5. The tissue was diluted 1:1 with the buffer solution and centrifuged by accelerating the rotor to 2200 x g and immediately decelerating it. The supernatant was collected and respun in the same manner after which it was again collected and spun at 2200 x g for 15 min. The supernatant was then discarded and the resulting "double-layered" pellet was resuspended in the same buffer as before, homogenized and again spun at 2200 x g for 15 min. The supernatant was discarded and the pellet was resuspended in a small amount of buffer solution. The suspension was then layered onto discontinuous gradients of sucrose in 0.01 M Tris, 0.001 M Na_2EDTA, pH 7.5. The gradients consisted of 39% sucrose (3 ml), 37% sucrose (6 ml), and 32% sucrose (2 ml). The membranes were then centrifuged at 75,000 x g for 90 min at 4°C. Following this there were major bands present at all interfaces as well as a pellet. The small amount of protein that doesn't enter

the sucrose and the material found at the 37% sucrose interface have identical PTH-stimulated adenylate cyclase activity and binding characteristics, so the two were combined by aspirating with a Pasteur pipette. The material at the 39% interface binds PTH very poorly and was discarded. The combined materials from the 32% and 37% interfaces were diluted in three volumes of a buffer solution consisting of 0.01 M Tris, 0.001 M Na_2EDTA, pH 7.5, and then centrifuged at 7800 x g for 15 min to remove the sucrose. The pellet was then resuspended in 0.05 M Tris, pH 7.5 and split up into 1 ml aliquots which were centrifuged at 2200 x g for 15 min, the supernatants discarded, and the pellets stored at -70°C.

Dog Renal Adenylate Cyclase Assay. The incubation mixture consisted of the following: 0.05 M Tris-HCl, 0.84 mM ATP, 0.08 to 2.0 x 10^6 cpm of [α-^{32}P]ATP, 9 mM theophylline, 4.2 mM $MgCl_2$, 26 mM KCl, 0.118% (w/v) bovine serum albumin, 5 mM creatine phosphate (Schwarz/Mann Division, Becton-Dickenson & Co.), 0.1 mg/ml of creatine phosphokinase, and 40 to 90 ug of membrane protein (53). FNPA-^{127}I-[Nle^8, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide was added in 5 ul and the total volume was brought to 100 ul. Incubations were started by addition of the membrane suspension and run for 10 min at 37°C. Termination was accomplished by the addition of 100 ul of a solution consisting of 0.125 M unlabeled cAMP, 0.04 M ATP, and approximately 20,000 cpm of [^{3}H]-cAMP in 0.05 mM Tris-HCl at pH 7.4. The reaction mixture was then boiled for three minutes to assure termination. The [^{32}P]-cAMP generated was then separated from the other [^{32}P]-containing reaction by-products through ion-exchange chromatography on columns of Dowex 50 W-X4 (H^+ form; 200-400 mesh, J.T. Baker Chemical Co.) followed by columns of alumina. Protein concentration was determined by the method of Lowry et al

(23) with bovine serum albumin as standard.

The bPTH standard used in the assays was Medical Research Council Standard, lot no. 76/572. Each peptide was assayed twice at multiple concentrations.

Photoreaction of Membranes and Photolabile Analogues. 100 ug aliquots of dog renal membrane were suspended in 100 ul of a reaction buffer consisting of 0.05 mM Tris-HCl, 9 mM theophylline, 4.2 mM $MgCl_2$, 26 mM KCl, 0.118% (w/v) bovine serum albumin, and placed in 12 x 75 mm glass test tubes in a 15°C water bath. To the tubes which were to contain competing peptides, 10 ug of the appropriate peptide was added and preincubated for ten minutes. Total volume of these tubes was then brought to 200 ul by addition of 100 ul of buffer solution. In the dark, 1.2×10^6 cpm of radioactive photoaffinity-labelled peptide was added to each test tube and incubated for 45 min. The test tubes were vortexed gently every 15 min. The total volume of the remaining tubes was brought to 200 ul and all the tubes were then exposed for 20 min to a high-pressure mercury vapor lamp at 12 inches. After centrifuging the tubes at 2250 x g, 4°C for 4 min, the supernatant was aspirated. The pellets were resuspended in a solution consisting of 75 ul water, 25 ul 10% SDS, and 25 ul of a sample buffer. The sample buffer consisted of 5 ml glycerol, 1 g SDS, 2.5 ml β-mercaptoethanol, 0.25 ml of 0.2% bromphenol blue in double distilled water and 2.5 ml of a stock solution made by dissolving 3 g Tris-Base in a total volume of 25 ml of water, pH 6.8. The suspensions were then boiled for 2 min and mounted immediately on SDS-acrylamide gels.

Electrophoresis in Sodium Dodecyl Sulfate-Polyacrylamide Gels. SDS-acrylamide 9 - 20% gradient gels (10 x 15 x 0.15 cm) were prepared by the

method of Laemmli (22). 25 ul aliquots of the membrane suspensions were mounted in each lane. Marker proteins were run in separate lanes. The SDS-acrylamide gels were run at constant voltage till the bromphenol blue stacking line reached the bottom. After electrophoresis the gel was stained with Coomassie blue and destained, then dried down and mounted for radioautography on Kodak X-Ray Film Type SB-5.

Results

Analytical Data. Data are presented for the preparative run of FNPA-^{127}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide used in the adenylate cyclase assay. HPLC was used to separate the photolabelling-reaction products. Two peaks from this HPLC consisted of FNPA-labelled peptide. Material from both peaks was analysed spectroscopically and the azide group's absorptions were normalized with respect to the relative amounts of peptide bond absorptions in lieu of known ligand concentrations. A ratio of 2:1 was found, most probably corresponding to a like number of FNPA-lysine amino group bonds per peptide molecule (total of three lysines per peptide). Material from Peak 1 (the most abundant, with a single FNPA per molecule) was reinjected for an analytical HPLC run to check for impurities. (1)-FNPA-^{127}I-[Nle^{8}, Nle^{1}, Tyr^{34}]bPTH-(1-34)-amide is shown in Figure 16 from the analytical run. Heterogeneity was estimated as less than 1%, showing this peptide to be pure when used in the adenylate cyclase assay. Figure 16 is a composite HPLC chart to illustrate the relative elution points of [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide, ^{127}I-[Nle^{8}, Nle^{18}, Tyr^{34}] bPTH-(1-34)-amide, and the FNPA-^{127}I-labelled peptide.

Bioactivity. Both peaks from the preparative HPLC were assayed twice

in the dog renal adenylate cyclase assay. Calculated potencies for (1)-FNPA-^{127}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide were 24,300 MRC units/mg and 30,800 MRC units/mg. With a mean potency of 26,000 MRC units/mg (95% confidence limits: 22,000 - 30,800), this photolabile PTH analogue is quite active. In fact these preliminary figures make it the most potent PTH analogue yet made†. Potency values for (2)-FNPA-^{127}I- [Nle^{8}, Nle^{18}, Tyr^{34}]-bPTH-(1-34)-amide were 5600 MRC units/mg and 11,600 MRC units/mg respectively. Though not as potent as (1)-FNPA, (2)-FNPA, with two FNPA-lysine amino bonds, is still more potent than the native hormone bPTH-(1-84).

SDS-Acrylamide Gel Radioautographs. Coomasie blue staining of the gels reveal many protein bands. Of these, there are five to six distinct bands visible on the radioautograph, Figure 17. Lane 1 consists of the membrane/photoligand reaction without competition. Lane 2 is with 10 ug [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide as competition. One band disappears in Lane 2. Using the known weights of the marker proteins, the weight of any unknown protein band can be calculated as follows: for the ratio **d/L** where **L** is the gel lane length and **d** is the distance traveled through the gel by the protein band, a plot of the log of the molecular weight vs **d/L** will be linear. The **d/L** of the unknown is plotted along the "known" graph to find the molecular weight. Figure 18 shows the line plots for two gels; one with FNPA-^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide and the other with SMHA-^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide. In both cases the weight of the unknown band which competes out is 70,000 $\pm$ 1000 Daltons. This same band is extinguished with the addition of inhibitor but is unaffected by inactive fragments of bPTH.

†[Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide held that distinction in Section I with an **in vitro** biopotency of 24,000 MRC units/mg.

Discussion

Receptors have long been studied at arm's length by manipulating the hormones that interact with them, the cells that sprout them, and their working conditions. With the recent advent of labelling techniques capable of tagging a receptor, the research takes on the new emphasis of actually finding the receptor.

For parathyroid hormone a photoactive group was chosen for its advantages in activation and controllability. In selecting the photoactive group, the aryl azides were among the most commonly used in other hormone photolabelling systems(1,6). The attributes of the azide moiety include a fast conversion to the reactive intermediary, the nitrene, and a lack of intramolecular rearrangements to less reactive forms. These two factors are crucial in that they determine the efficiency of the reaction and, ultimately, the reproducibility of the results. Fast conversion also means less exposure to the intense irradiation that may damage other components of the photolabelling system. The nitrene intermediary is capable of reacting with all types of carbon-hydrogen bonds and hydroxyl bonds, all of which are abundant in a protein receptor. 4-Fluoro-3-Nitrophenyl Azide (FNPA) and N-Succinimidyl-6(4'-Azido-2'-Nitrophenyl Amino) Hexanoate (SMHA) were chosen for their ease of attachment. Both react with primary amines under relatively mild conditions. The two groups had advantages and disadvantages. Succinimide being the better leaving group, SMHA attachment to bPTH was more certain than FNPA but its hexanoate group was much bulkier than the phenyl ring of FNPA and this was a possible liability in producing a bioactive PTH ligand. To increase the chances of success, both were synthesized. In the event that both ligands were active they should label the same membrane components, bolstering the confidence in the labelling

obtained.

With the choice of photolabile group made, it remained to be proven that it would attach to PTH and, once attached, would not interfere with PTH's bioactivity, **re** specificity for the receptor. A scaled-up synthesis of FNPA-^{127}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide was made as a representative check for azide incorporation and bioactivity. After completion of the synthesis, the entire reaction mixture was injected in an HPLC and the peaks were collected in the dark. Two large peaks, close together and early in the reverse-phase run, were collected separately and found to contain peptide with FNPA. To demonstrate the homogeneity of the material used in the **in vitro** assay an analytical HPLC run was performed on material from the first peak (Figure 16). The peptides were analyzed spectroscopically by hand. Since both peptides had the same molecular extinction coefficients for the amino acid backbone the relative concentrations of the two samples could be used to normalize the azide absorptions. When this was done, the second peak was found to have twice the azide absorbance per molecule of peptide peak 1. In the bPTH 1 to 34 sequence there are only four possible attachment points: the α-amino group on the position 1 alanine and the ϵ-amino group on the three lysines (positions 13, 26, and 27). Amino acid analysis was not helpful in ruling out the position 1 alanine since the FNPA-amino bond proved to be labile under the conditions employed (5.7 N HCl at 110°C for 24 h), but an FNPA-Ala^{1} attachment was considered unlikely in this material due to its enhanced bioactivity. Any deliberate modifications of position 1 have decreased or destroyed the potency of PTH-(1-34) in the past (57). With three lysines, in each peptide a 1:2 ratio of absorbances suggests 1 FNPA/peptide and 2 FNPA/peptide for (1)-FNPA-^{127}I-bPTH and (2)-FNPA-^{127}I-bPTH respectively.

Edman sequencing may prove useful in finding which positions were substituted. In the **in vitro** dog renal adenylate cyclase assay (1)-FNPA-^{127}I-bPTH had a markedly increased potency of 30,800 MRC units/mg in the first assay and 24,300 MRC units/mg in the second. While not nearly as active, (2)-FNPA-^{127}I-bPTH was still more potent than unsubstituted bPTH-(1-34). A similar preparation and analysis was not done with SMHA-bPTH. Since the radioactive preparation labels the same bands as FNPA it must be assumed to have retained its biological activity also.

A slightly different protocol was used in the preparation of the ^{125}I labelled photolabile PTH analogues because of the small amounts of peptide used (2 ug) and the radioactivity. QUSO-32 was used to separate ^{125}I-PTH after iodination instead of a P-2 column. More significant a change was the use of the reaction mixture from the photolabelling without purification. Since the unreacted and partially reacted by-products would lack the crucial combination of both photolabel and ^{125}I and thus would not affect the radioautographic bandings or the specificity of the FNPA and SMHA ^{125}I labelled analogues, purification was not necessary.

The biospecificity of the analogues was checked by using both competitive antagonists and agonists with inactive fragments as controls. These included [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide (agonist), [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(3-34)-amide (antagonist), bPTH-(53-84) (approximate size inactive fragment), and bPTH-(1-12) with bPTH-(13-34) (full sequence in fragments). Of the many protein bands on the Coomasie blue-stained gel, only four to six were distinctly labelled on the radioautograph. One band, at molecular weight 70,000, disappeared when competing amounts of agonist were added. This same band was unaffected by inactive fragments of bPTH

but was eliminated by antagonist as well. In addition, porcine renal cortical membranes (cell line LLC-PK_1), which were known to be responsive to calcitonin but unresponsive to PTH (13), were labelled resulting in a different radioautographic pattern almost devoid of bands which was unchanged with addition of agonist and lacked a 70,000 weight band. Non-specific binding of the ligand to albumin, which is in the same weight range and present in the membrane preparation, was also ruled out by labelling heat-inactivated serum albumin and running it in a separate lane on the gel.

From the evidence available, it appears that the labelled band at 70,000 molecular weight is the parathyroid hormone receptor or at least a receptor subunit. The 9 - 20% SDS-acrylamide gel used is generally capable of resolving proteins in the 10-15,000 to 120,000 weight range. Within that range there do not appear to be any other competable bands. To be more complete, other gels should be made to look for protein bands at higher and lower weights as well as improve the resolution at the upper and lower limits of the 9 - 20% gels. Taking another approach, a photolabile bPTH-(1-84)-type, if it could be made, might label a different receptor subunit. The other bands evident on the radioautograph represent some form of non-specific binding since they are not affected by competition, but what is especially interesting is the constancy of the pattern. They may prove to be membrane proteins in close approximation to the PTH receptor.

The much enhanced potency of (1)-FNPA^{125}I-PTH was a serendipitous discovery. Modification of the lysine ϵ-amino groups is apparently a fertile area for the design of super-potent PTH agonists. The group Fluoro-2-Nitrobenzene, FNPA without the azide moiety, would be a good first choice to follow this up.

The entire receptor complex for parathyroid hormone will undoubtedly turn out to be very complicated. This work is just a beginning in studies on the receptor proper and as with most beginnings it raises many questions. The development of photolabile ^{125}I-labelled PTH analogues is an important step. Aside from the question of whether they have identified receptor or receptor subunit, their biological activity **in vitro** and their specificity for certain membrane proteins in dog renal membranes have been demonstrated. Of immediate use is their role as probes in other tissues where their labelling of similar protein(s) or entirely different protein(s) would have important implications. As for answering the question of receptor vs. receptor subunit, purification and study of the 70,000 molecular weight band may help and is one of the next steps to pursue.

Table I: Amino Acid Content of Synthetic Peptides§

residue	predicted	[D-Tyr^{34}]bPTH-(1-34)-amide obtained	[D-Val^{2}, D-Tyr^{34}]bPTH-(1-34)-amide obtained	predicted	[D-Tyr^{34}]bPTH (2-34)-amide obtained	[D-Val^{2}, D-Tyr^{34}]bPTH-(2-34)-amide obtained
Asp	3	3.2	3.3	3	3.1	3.1
Ser†	3	2.8	3.1	3	2.8	2.8
Glu	5	5.3	5.4	5	5.1	5.0
Gly	1	1.1	1.2	1	1.2	1.1
Ala	1	0.8	0.9	0	<0.1	<0.1
Val	3	2.9	3.0	3	2.8	2.8
Met	2	1.9	2.0	2	1.8	2.0
Ile	1	0.9	0.9	1	0.9	0.9
Leu	4	4.2	4.4	4	4.2	4.2
Tyr	1	0.9	0.9	1	1.0	1.0
Phe	1	0.8	0.7	1	1.0	1.0
Lys	3	3.2	3.2	3	3.2	3.2
His	3	3.0	3.0	3	3.0	3.1
Arg	2	2.1	2.1	2	2.1	2.1

§ All values represent the average of three separate aliquots of peptide after acid hydrolysis. Tryptophan content was not determined. †Corrected for degradative losses during acid hydrolysis.

Table II: Biological activity of Bovine Parathyroid Hormone Analogues in Rat Renal Adenylate Cyclase Assay

substitution	fragment length	combined potency estimate[a] (MRC units/mg)	relative potency[b] (%)
none	1-34[c]	5400 (3900-8000)	100
[Tyr^{34}]	1-34 amide[c]	16000 (11000-23000)	300
[D-Tyr^{34}]	1-34 amide	14500 (11000-17000)	270
[D-Tyr^{34}]	2-34 amide	130[d](120-150)	3
[D-Val^{2}, D-Tyr^{34}]	2-34 amide	90[d](60-100)	2
[D-Val^{2}, D-Tyr^{34}]	1-34 amide	80[d](60-100)	1

[a]Combined potency estimate based on three independent assays except for the compound [D-Tyr^{34}]bPTH-(1-34)-amide. Limits in parenthesis represent standard error of the mean for each of the peptides, except for [D-Tyr^{34}]bPTH-(1-34)-amide, for which 95% confidence limits are provided. Peptides were assayed without prior treatment with reducing agents. [b]Relative potency calculated on the basis of the mean potency with the activity of the reference peptide, unsubstituted bPTH(1-34), taken as 100%. [c]Potencies previously reported (Potts et al., 1971; Rosenblatt & Potts, 1977). [d]Response curve nonparallel to that of the standard. Although the potency value cannot be formally assigned, potency has been estimated by comparing the activity with that of the standard at half-maximal stimulation by the analogue

Table III: Amino Acid Content of Sulfur-Free Synthetic Peptides§

residue	predicted	[Nle^{8}, Nle^{18}, [D-Tyr^{34}]bPTH-(1-34)-amide obtained	[D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide obtained	predicted	[D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide obtained
Asp	3	3.1	3.2	3	3.3
Ser†	3	2.9	2.9	3	2.8
Glu	5	4.9	5.1	5	5.2
Gly	1	1.3	1.1	1	1.1
Ala	1	1.0	1.0	0	<0.1
Val	3	3.1	3.1	3	2.9
Nle	2	1.7	1.8	2	1.8
Ile	1	1.5	1.2	1	1.1
Leu	4	4.2	4.4	4	4.5
Tyr	1	0.9	0.9	1	0.9
Phe	1	0.8	0.8	1	0.8
Lys	3	3.2	3.2	3	3.1
His	3	3.0	3.0	3	2.8
Arg	2	2.1	2.4	2	2.2

§ All values represent the average of a minimum of two separate aliquots of peptide after acid hydrolysis. Tryptophan content was not determined. †Corrected for degradative loss during acid hydrolysis.

Table IV: Biological Activity of Sulfur-Free Bovine Parathyroid Hormone Analogues

substitution	fragment length	**In Vitro** Rat Renal Adenylate Cyclase Assay: combined potency estimate[a] (MRC units/mg)	**In Vitro** Rat Renal Adenylate Cyclase Assay: relative potency[b] (%)	**In Vivo** Chick Hypercalcemia Assay: potency[a] (MRC units/mg)	**In Vivo** Chick Hypercalcemia Assay: relative potency[b] (%)
none	1-34[c]	5400 (3900-8000)	100	7700	100
[D-Tyr^{34}]	1-34 amide[c]	14500 (11000-17000)	270	not done	
[Nle^{8}, Nle^{18} D-Tyr^{34}]	1-34 amide	24000 (21000-28000)	440	2600	34
[D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]	1-34 amide	50[d]	2	<100	<1
[D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]	2-34 amide	30[d]	1	170	2

[a] Combined potency estimate based on at least two independently valid and statistically homogeneous assays. Limits in parenthesis represent standard error of the mean for each of the peptides. [b]Relative potency calculated on the basis of the mean potency with the activity of the reference peptide, unsubstituted bPTH(1-34), taken as 100%. [c]Potencies from Part I. [d]Response curve nonparallel to that of the standard. Although the potency value cannot be formally assigned, potency has been estimated by comparing the activity with that of the standard at half-maximal stimulation by the analogue.

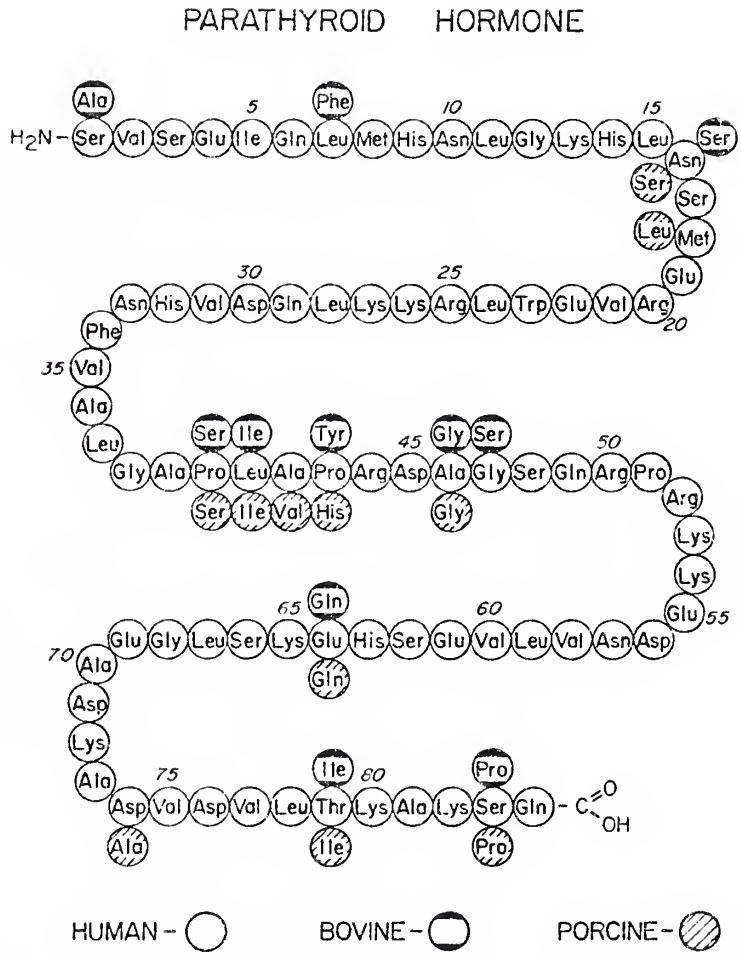


Figure 1. The 84 amino acid sequence of human parathyroid hormone (hPTH) and the variations of bovine (bPTH) and porcine (pPTH) parathyroid hormones.

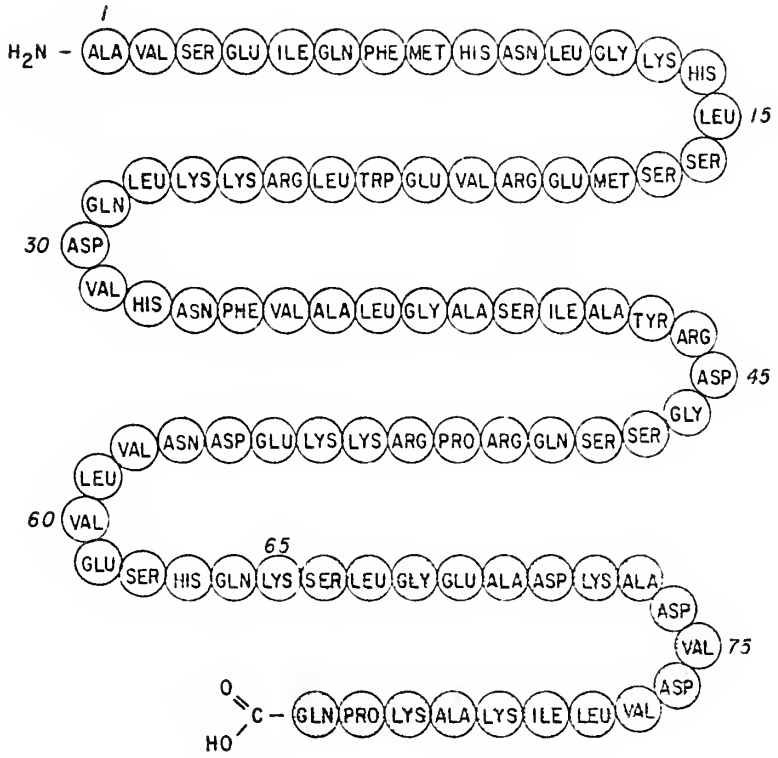


Figure 2. The full sequence of bovine parathyroid hormone, bPTH-(1-84).

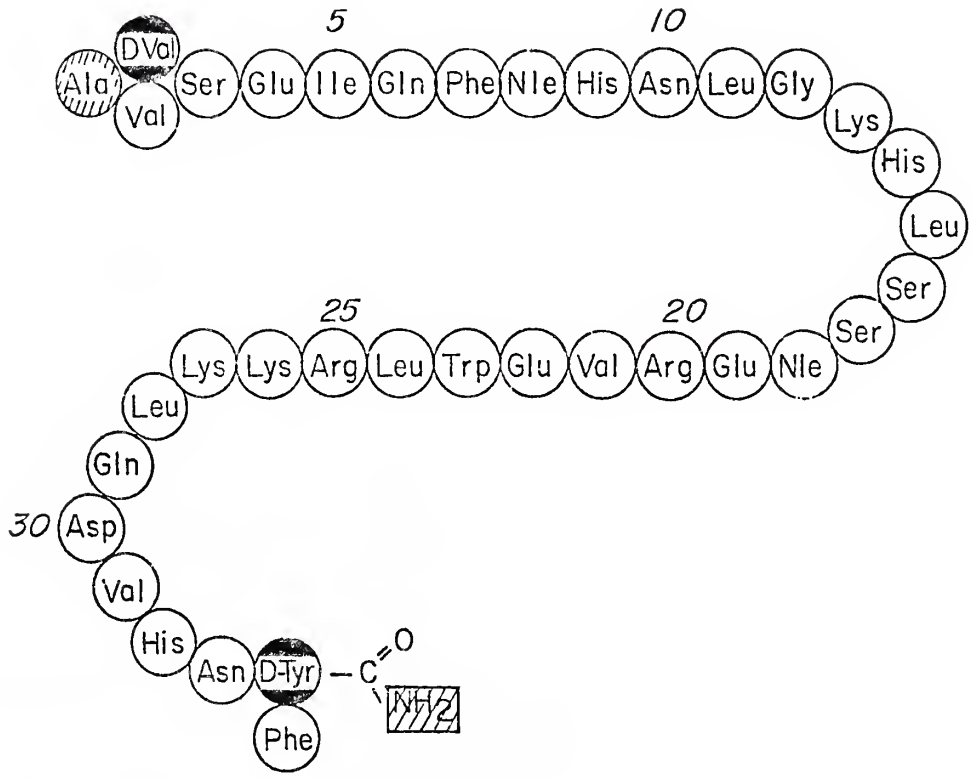


Figure 3. Sequence of the four D-amino acid-containing analogues. All four contain the carboxyamide function and the D-tyrosine substitution for phenylalanine. In two of the analogues, D-valine was substituted for the L-valine at position 2. Additionally, two of the analogues had position 1 deleted. The four analogues are [D-Tyr^{34}]bPTH-(1-34)-amide, [D-Val^{2}, D-Tyr^{34}]bPTH-(1-34)-amide, [D-Tyr^{34}]bPTH-(2-34)-amide, [D-Val^{2}, D-Tyr^{34}]bPTH-(2-34)-amide.

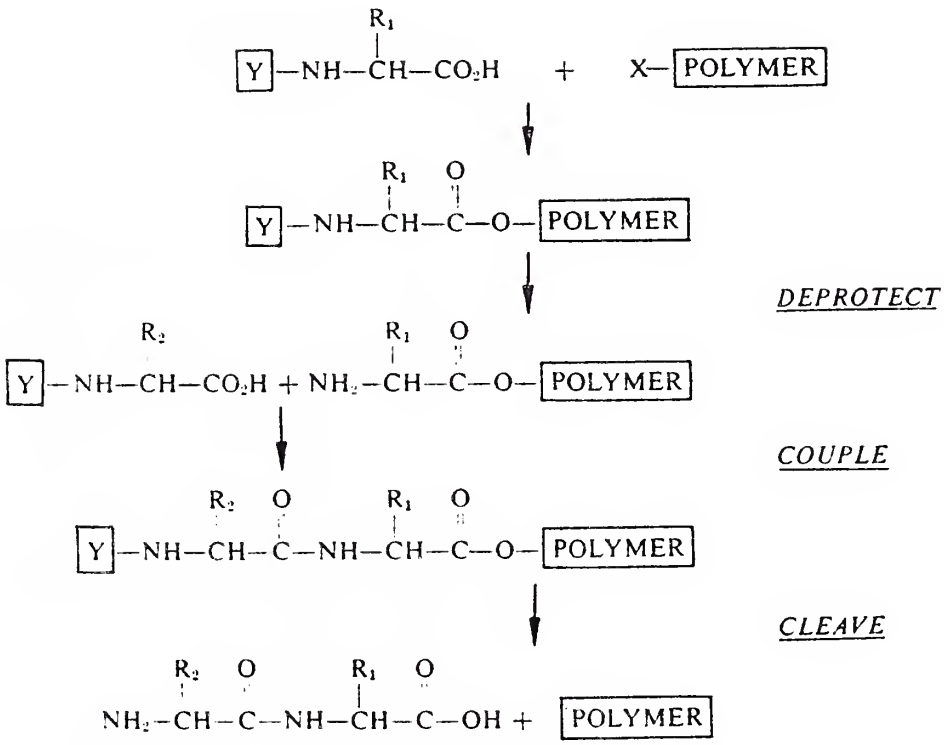


Figure 4. Schematic of Merrifield solid-support synthesis of a two-amino acid peptide. The deprotect and couple steps are repeated as many times as needed to elongate the peptide.

	Peptide Sequence							Content %
Major Peptide	A - B - C - D - E - F - G - H							85
Error Peptides								
- deletion of C	A - B - D - E - F - G - H -							5
- deletion of E	A - B - C - D - F - G - H -							5
- deletion of G	A - B - C - D - E - F - H -							5
	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7	
Edman Yields %	A 100	B 100	C 95 D 5	D 95 E 5	E 90 F 10	F 90 G 10	G 85 H 15	

Figure 5. Edman degradation performed on a peptide ABCD...which is contaminated by small amounts of deletion error-containing peptides results in a "preview" of the next amino acid. At the point of the deletion, the "preview" amino acid occurs in an amount corresponding to the number of deletion containing peptides. As the sequencing proceeds, the effect of successive deletions is cumulative resulting in an amplification of even a very small error.

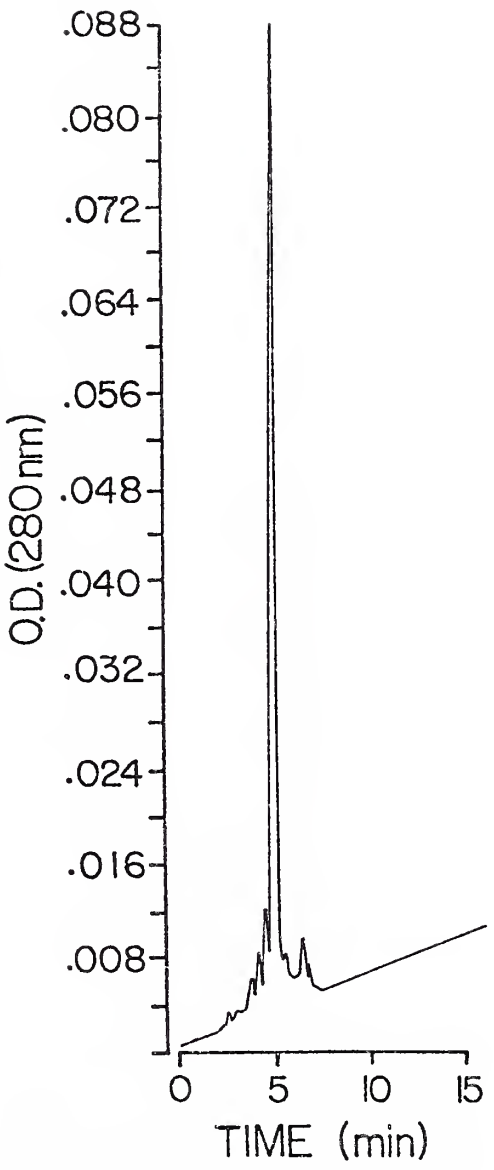


Figure 6. Reverse-phase high-pressure liquid chromatographic profile of [D-Val^{2}, D-Tyr^{34}]bPTH-(1-34)-amide, one of the four D-amino acid containing analogues derived from a single synthesis.

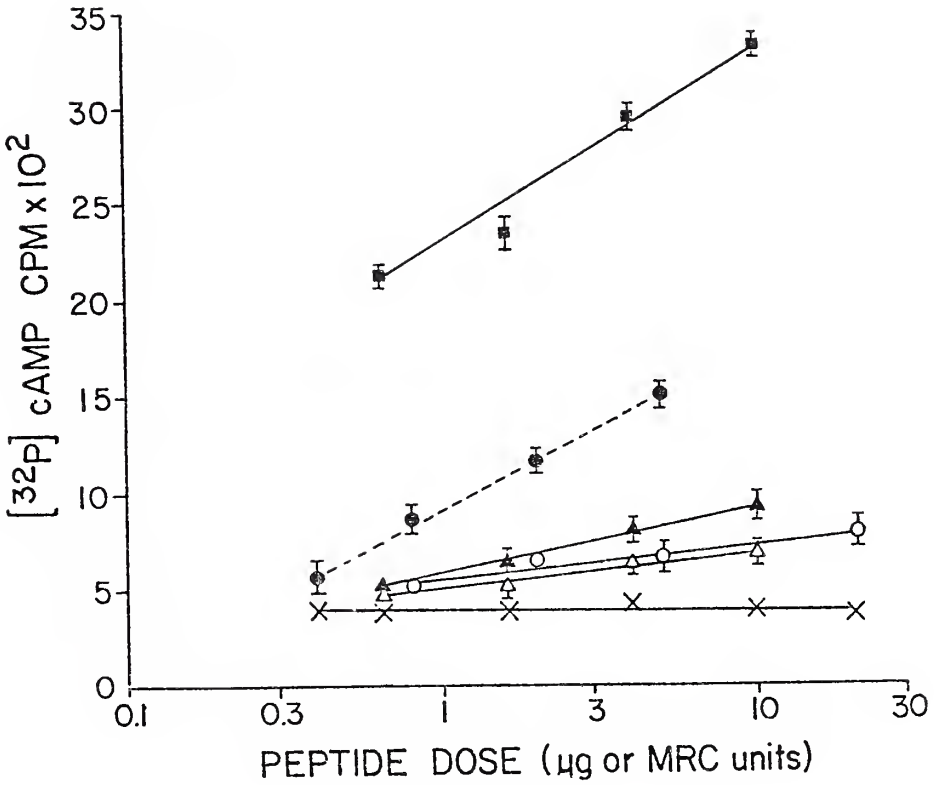


Figure 7. Composite of representative rat renal cortical adenylate cyclase assays of the following: [D-Tyr^{34}]bPTH-(1-34)-amide (■); native bovine hormone standard, bPTH-(1-84), 2500 MRC units/mg (●); [D-Tyr^{34}]bPTH-(2-34)-amide (▲); [D-Val^{2}, D-Tyr^{34}]bPTH-(2-34)-amide (○); [D-Val^{2}, D-Tyr^{34}] bPTH-(1-34)-amide (△); [D-Tyr^{34}]bPTH-(1-34)-amide (×), assayed 18 weeks after purification was completed. Each point is the mean of triplicate determinations. Peptide concentration was quantitated by amino acid analysis. Each peptide was assayed within 4 weeks of completion of purification without prior treatment with reducing agents.

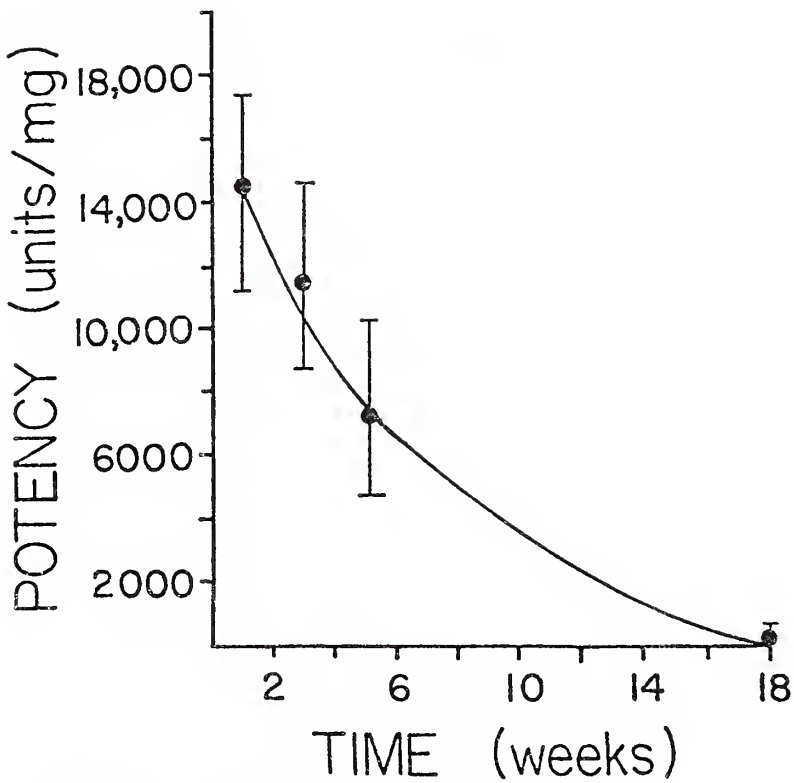


Figure 8. Biological activity of [D-Tyr^{34}]bPTH-(1-34)-amide vs. time elapsed from completion of synthesis. Bioactivity was determined without prior treatment with reducing agents. Error bars depict standard error of the mean.

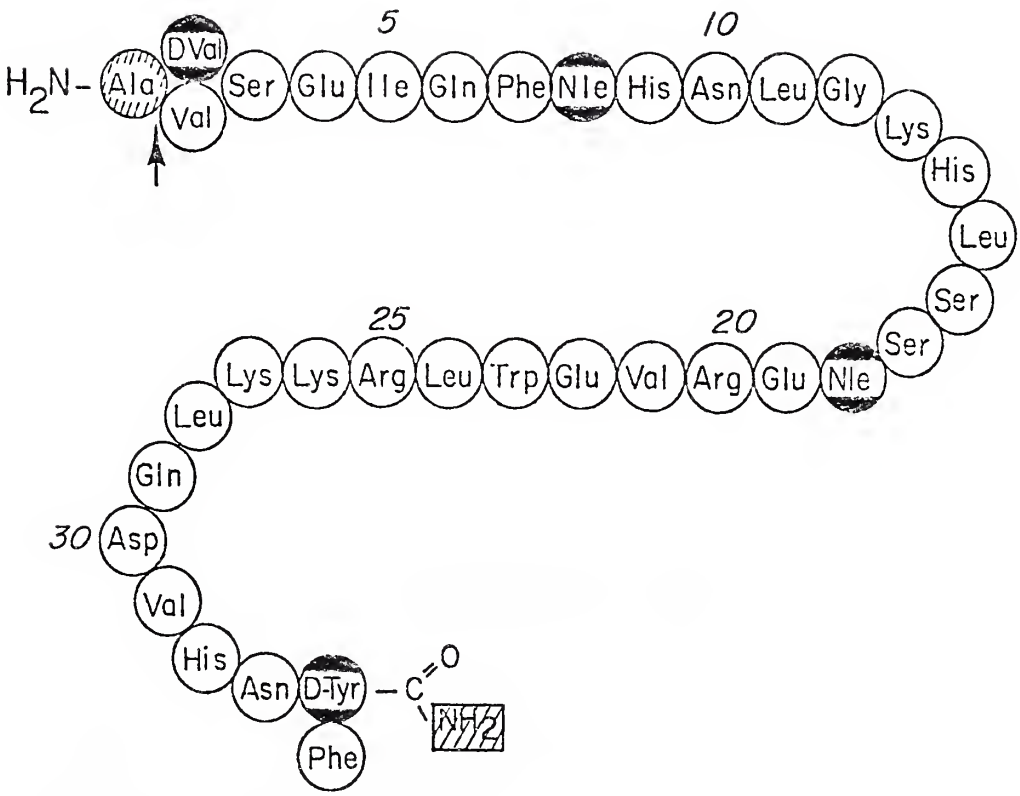


Figure 9. Sequence of the three D-amino acid-containing sulfur-free analogues. All three contain the carboxyamide function and the D-tyrosine substitution for phenylalanine. In two of the analogues, D-valine was substituted for the L-valine at position 2. Additionally, in one of the analogues position 1 was deleted (arrow). The three analogues are [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, and [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide.

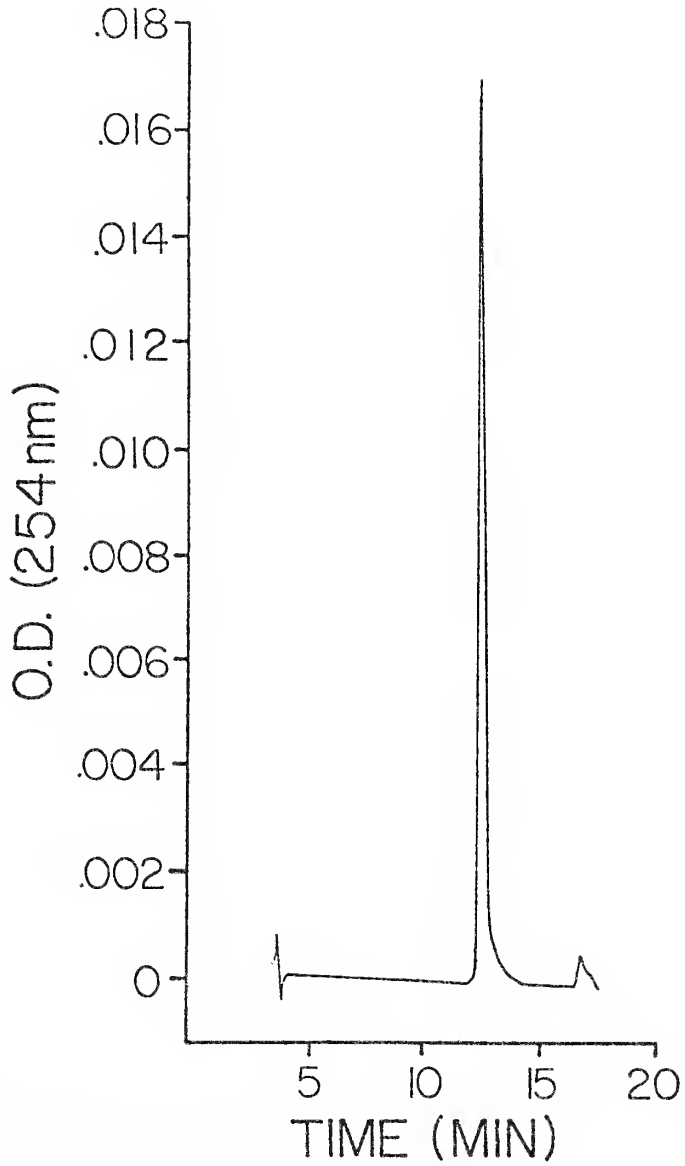


Figure 10. Reverse-phase high-pressure liquid chromatographic profile of [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, one of the three D-amino acid-containing sulfur-free analogues derived from a single synthesis.

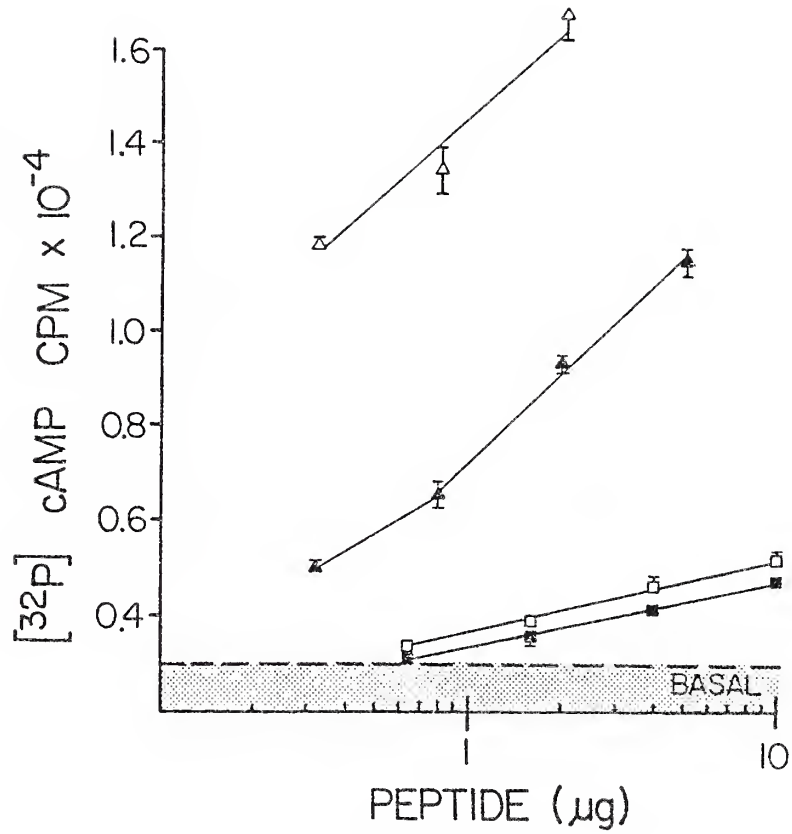


Figure 11. Composite of representative rat renal cortical adenylate cyclase assays of the following: [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide (△); native bovine hormone standard, bPTH-(1-84), 2500 MRC units/mg (▲); [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide (□); [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}] bPTH-(2-34)-amide (■). Each point is the mean of triplicate determinations. Peptide concentration was quantitated by amino acid analysis.

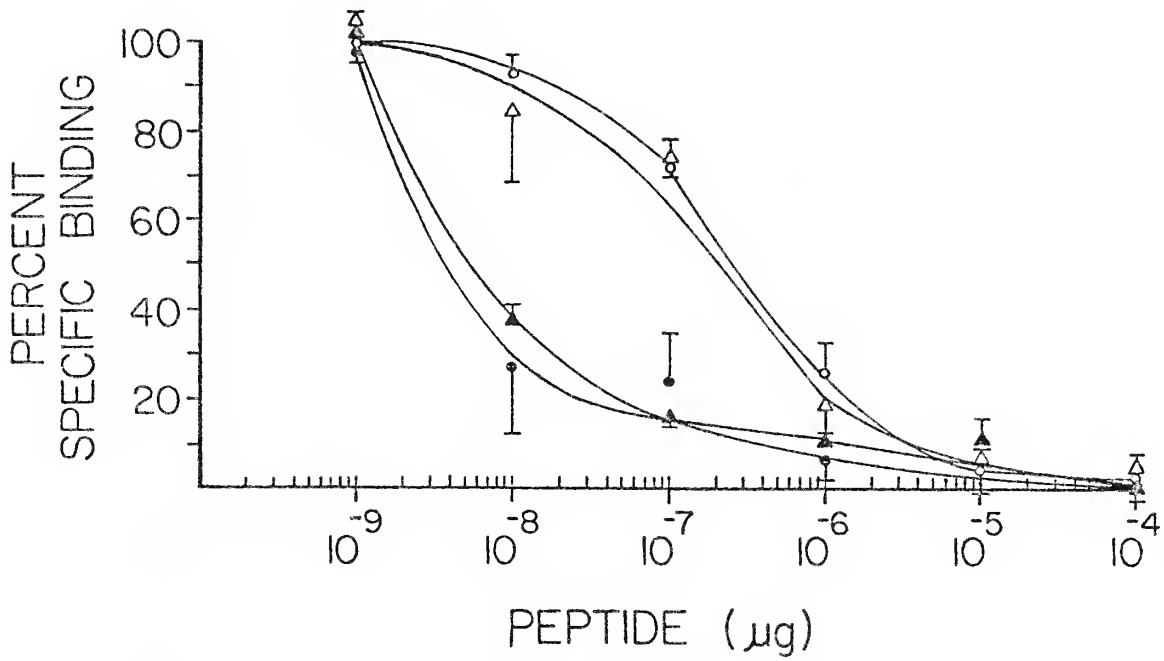


Figure 12. Binding properties of four analogues of PTH in a canine renal radioreceptor assay for PTH. ^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide was used as the radioligand. Inhibition of the radioligand specific-binding is shown by (●) for [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide, by (▲) for [Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide, by (△) for [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(1-34)-amide and by (○) for [D-Val^{2}, Nle^{8}, Nle^{18}, D-Tyr^{34}]bPTH-(2-34)-amide. Each point is the mean of triplicate determinations. Error bars depict standard error of the mean.

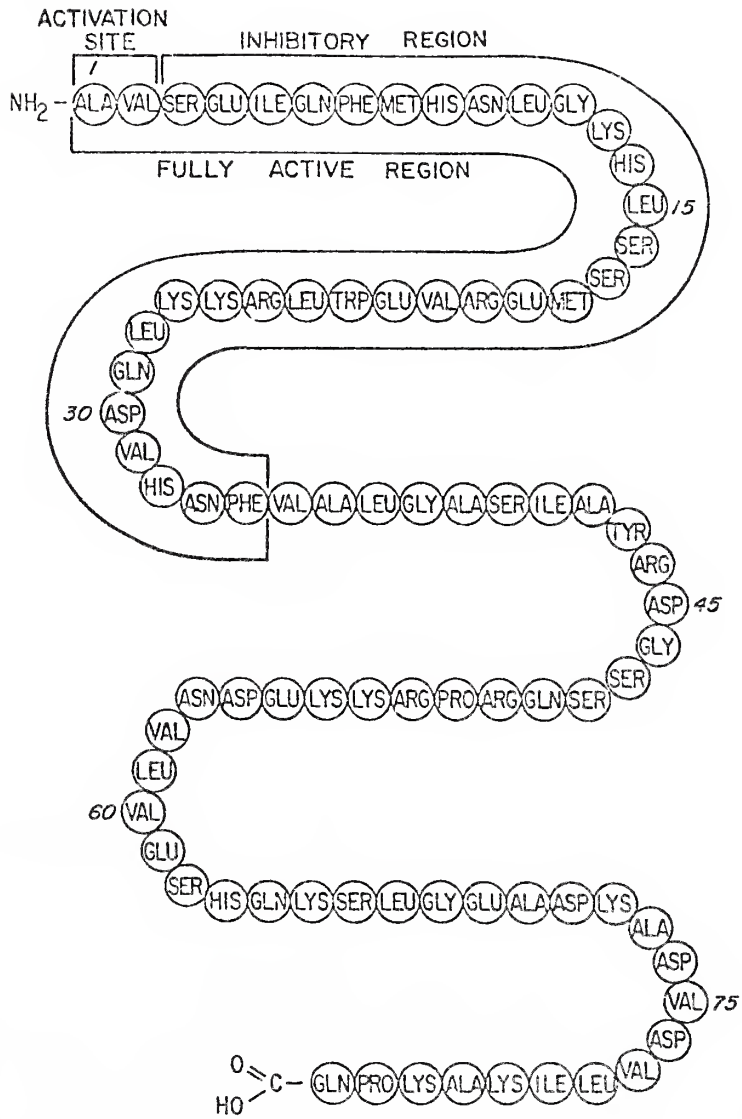


Figure 13. The full sequence of bPTH-(1-84). The fully biologically active region 1 to 34 is enclosed in brackets. This region is further resolvable into 1) an amino-terminus region (positions 1 and 2) that is essential for biological activity once binding to the receptor has occurred and 2) a region (positions 3 to 34) that binds the PTH receptor without activating adenylate cyclase.

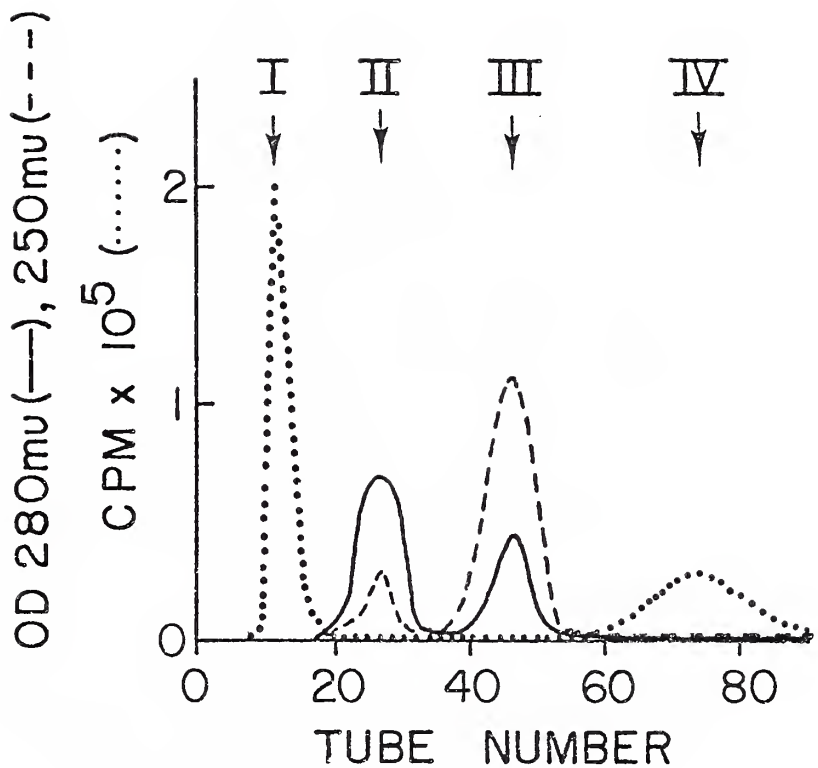


Figure 14. Elution profile of iodinated peptide after purification on a Bio-Gel P-2 column in 0.1 M HOAc. **Peak I**, labelled peptide; **Peak II**, chloramine-T; **Peak III**, sodium metabisulfite; **Peak IV**, iodine.

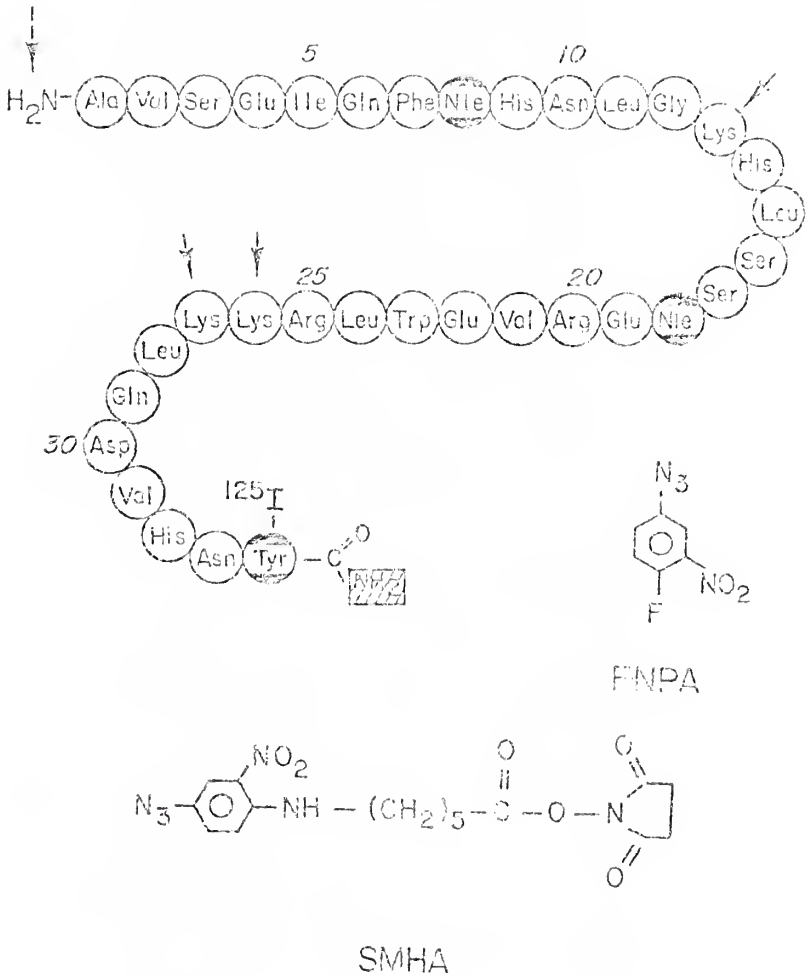


Figure 15. Primary structure of the photolabile PTH analogues. The analogues are sulfur-free versions to withstand the oxidative conditions needed to attach ^{125}I to the terminal tyrosine. Solid arrows point to the three lysine residues that the aryl azides may react with. The discontinuous arrow points to the amino group on alanine (see text). Also depicted are the two aryl azides used: FNPA, 4-Fluoro-3-Nitrophenyl azide and SMHA, N-Succinimidyl-6-(4'-Azido-2'-Nitrophenyl Amino) Hexanoate.

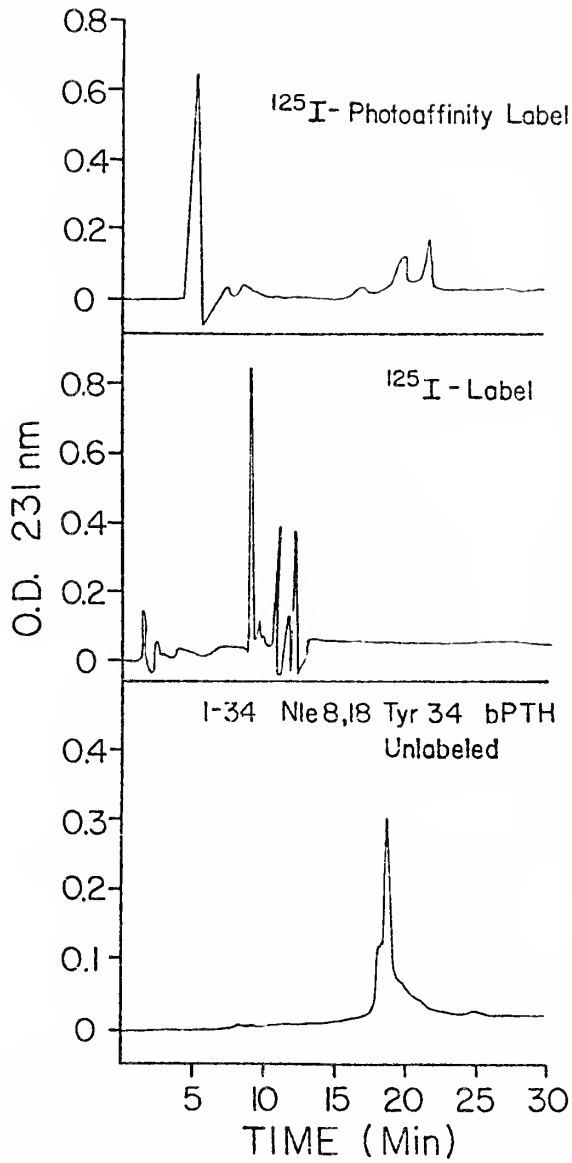


Figure 16. Composite HPLC of **top**, (1)-FNPA-^{127}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide; **center**, ^{127}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide; **bottom** [Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide. Buffers (as described in the text) were the same for all three, running on a linear 30 min (0-100% Buffer 2) gradient. For FNPA-bPTH, **top**, this analytical run shows little heterogeneity. Further, the two peaks corresponding to the unreacted ligands which would give an erroneous cyclase assay elute at different points and are not contaminants in the HPLC purified assay material.

Lane 1 Lane 2

Figure 17. Radioautograph of 9 - 20% SDS-Polyacrylamide gradient gel. Both lanes contain membrane and SMHA-^{125}I-[Nle^{8}, Nle^{18}, Tyr^{34}]bPTH-(1-34)-amide incubated and exposed to light as explained in the text. In addition, **Lane 2** contains 10 ug of unlabelled [Nle^{8}, Nle^{18}, Tyr^{34}]-bPTH-(1-34)-amide for competition. One band, corresponding to a molecular weight of 70,000 $\pm$ 1000 daltons, disappears from **Lane 2** (arrow).

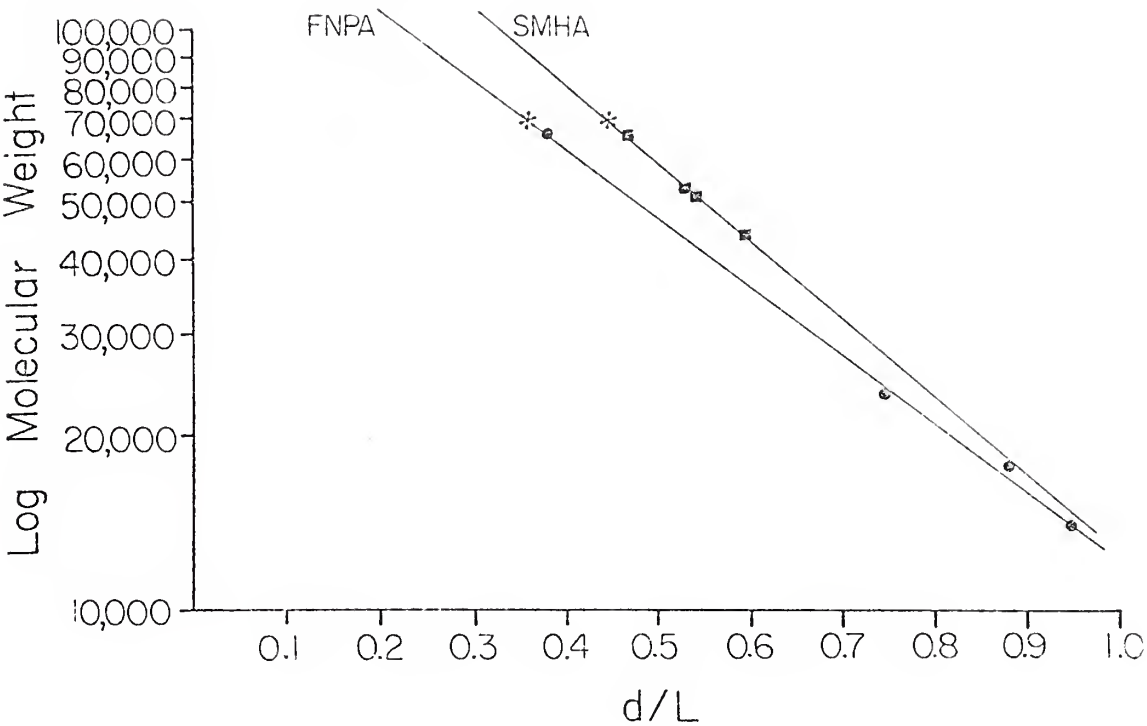


Figure 18. Straight-line functions based on **d/L** vs. log molecular weights of known proteins. The difference in plot slopes is due to the two separate gels represented. Gel 1 (●) was loaded with FNPA-labelled membranes and Gel 2 (■) was loaded with SMHA-labelled membranes. Both gels were 9 - 20% SDS-polyacrylamide gradients. The disappearance of the receptor/receptor subunit band corresponded to the same weight, 70,000 ± 1000 Daltons (astericks).

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